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SYNTHETIC LETHALITY OF CDK INHIBITION AND DOXORUBICIN IN TRIPLE-NEGATIVE BREAST CANCER REQUIRES P53 INACTIVATION

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DISSERTATION

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by
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DEDICATION

This dissertation is dedicated to my grandmother, Georget G. Stephan. As I have progressed through life, I have strived to be as strong, loving and generous as you always were. At times, however, the trait I best exemplify is your stubbornness. Fortunately, stubbornness and resilience often helped me endure all the highs and lows of graduate school. In times of self-doubt and exhaustion, I was reminded of your unyielding strength and how you never let illness hamper your daily life. You were never tired, never sad, constantly devoting your time to bettering the lives of others around you. If this project helps a fraction of the people you have impacted, it is a success. It is my hope, that with this project, I have helped spare other families from losing another grandmother, mother, daughter or friend. Thank you for all the encouragement, love and support. You are missed.

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SYNTHETIC LETHALITY OF CDK INHIBTION AND DOXORUBICIN IN TRIPLE-NEGATIVE BREAST CANCER REQUIRES P53 INACTIVATION

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Triple-negative breast cancer (TNBC) is an aggressive malignancy in which the tumors lack expression of estrogen receptor, progesterone receptor and HER2. As such, TNBC patients cannot benefit from clinically available targeted therapies and must rely on chemotherapy and surgery for treatment. While initially responding well to chemotherapy, TNBC patients are at increased risk of developing distant metastasis and have decreased overall survival compared to non-TNBC patients. A majority of TNBC tumors carry p53 mutations, enabling them to bypass the G1 checkpoint and complete the cell cycle even in the presence of DNA damage. Therefore, we **hypothesized that TNBC cells are sensitive to cell cycle targeted combination therapy, which leaves non-transformed cells unharmed**. Our findings demonstrate that sequential administration of the pan-CDK inhibitor roscovitine prior to doxorubicin treatment is synthetic lethal explicitly in TNBC cells. Furthermore, this novel combination therapy is well tolerated and efficacious, significantly reducing tumor volume and increasing overall survival compared to single drug treatment arms in a pre-clinical model system. Mechanistic studies found that combination treatment arrested TNBC cells in the G2/M cell cycle phase, where cells rely on homologous recombination for repair of DNA double strand breaks. Notably, combination treatment increased DNA double strand breaks, while simultaneously reducing recruitment of homologous recombination proteins. Examination of isogenic immortalized human mammary epithelial cells and isogenic tumor cell lines found that abolishment of the p53 pathway is required for combination-induced cytotoxicity; making mutated p53 a putative predictor of response to therapy. Consequently, p53 wildtype non-transformed cells are able to avoid cell death by arresting in G1. By exploiting the specific biological and

molecular characteristics of TNBC tumors, this innovative therapy has the potential to greatly impact the treatment and care of TNBC patients.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure

LIST OF TABLES

Table

ABBREVIATIONS

BMI	Body mass index
ER	Estrogen receptor
HBOC	Hereditary breast and ovarian cancer
DSB	Double strand break
HR	Homologous recombination
NHEJ	Non-homologous end joining
CDK	Cyclin dependent kinases
CKIs	CDK inhibitors
M	Mitosis
G1	Gap 1
S	DNA synthesis
G2	Gap 2
Rb	Retinoblastoma
HDAC	Histone deacetylase
CAK	CDK activating kinase
ATM	Ataxia telangiectasia
ATR	Rad3 related
SSBs	Single strand breaks
PIKKs	Phosphoinositide 3-kinase related kinases
BARD1	BRCA1-associated RING domain protein 1
pre-RC	Pre-replicative complex
ORC	Origin recognition complex
RPA	Replication protein A
Pol	Polymerases
PCNA	Proliferating cell nuclear antigen
Plk1	Polo like kinase 1
APC	Anaphase-promoting complex
FBXW7	F-box and WD repeat domain 7
GSK3β	Glycogen synthase kinase 3β
LMW-E	Low molecular weight cyclin E
GOF	Gain of function
EGFR	Epidermal growth factor receptor
ROS	Reactive oxidative species
ATRIP	ATR-interacting protein
TopBP1	Topoisomerase II binding protein 1
BER	Base excision repair
NER	Nucleotide excision repair
MMR	Mismatch repair
PARP	Poly (ADP-ribose) polymerase
TCNER	Transcription-coupled NER
TFIIH	Transcription factor IIH
RFC	Replication factor C
IDL	Insertion/deletion loops
MSH	MutS homologs
MutSα	MSH2-MSH6
MutSβ	MSH2-MSH3
MutLα	MLH1-PMS2
EXO1	Exonuclease 1
Mre11	Meiotic recombination 11
NBS1	Nijmegen Breakage Syndrome 1

MRN	Rad50/ meiotic recombination 11/ Nijmegen Breakage Syndrome 1
BRCT	BRCA-1 C-terminus
MDC1	Mediator damage checkpoint protein
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
CtIP	CtBP-interacting protein
BLM	Bloom's syndrome protein
dsDNA	Double stranded DNA
D-loop	Displacement loop
SDSA	Synthesis-dependent strand annealing
DSBR	Double strand break repair
HJs	Holliday junctions
ICLs	Interstrand crosslinks
FA	Fanconi anemia
BTR	Bloom's syndrome complex
DDR	DNA damage response
XP	Xeroderma pigmentosum
SNPs	Single nucleotide polymorphisms
NSCLC	Non-small cell lung cancer
HNPCC	Hereditary nonpolyposis colorectal cancer
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
PR	Progesterone receptor
SERMs	Selective estrogen response modulators
TTP	Time to progression
MBC	Metastatic breast cancer
DFS	Disease-free survival
RS	Recurrence score
CMF	Cyclophosphamide, methotrexate and fluorouracil
CAF	Cyclophosphamide, Adriamycin and fluorouracil
TAC	Docetaxel, doxorubicin (Adriamycin) and cyclophosphamide
PFS	Progression free survival by four months
FGFR	Fibroblast growth factor receptor
EGF	Epidermal growth factor
MAPK	Mitogen-activated protein kinase
RR	Response rate
pCR	Pathological complete response
BLBC	Basal-like breast cancer
CK	cytokeratin
TNBC	Triple-negative breast cancer
BL1	Basal like 1
BL2	Basal like 2
IM	Immunomodulatory
M	Mesenchymal
MSL	Mesenchymal stem-like
LAR	Luminal androgen receptor
MES	Mesenchymal
BLIS	Basal-like immune suppressed
BLIA	Basal-like immune activated
FGFR2	Fibroblast growth factor receptor 2
FAC	5-fluorauracil, doxorubicin and cyclophosphamide
VEGF	Vascular endothelial growth factor

VEGFR	Vascular endothelial growth factor receptor
SD	Stable disease
MTD	Maximum tolerated dose
TTP	Time to progression
IR	Ionizing radiation
CNDAC	2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine
HMEC	Human mammary epithelial
ATCC	American Type Cell Culture Collection
DMEM	Dulbecco's modified Eagle medium ()
FCS	Fetal calf serum
DMSO	Dimethyl sulfoxide
PBS	Phosphate-buffered saline
HTSA	High-throughput survival assay
IC	Inhibitory concentration
MTT	(3-(4, 5)-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
CI	Combination index
PI	Propidium iodide
RH	Relative hazard ratio
p53BP1	p53 binding protein
MMEJ	Microhomology-mediated end joining

CHAPTER 1: INTRODUCTION

1.1 EPIDIEMOLGOY AND RISK FACTORS OF BREAST CANCER

Although the rate of cancer mortality has decreased 20% over the past two decades, it is expected that over half a million people will die from cancer in the United States in the year 2015 [1]. Second only to lung cancer, breast cancer kills over 40,000 women each year [1, 2]. While 1 in 8 women will develop breast cancer in their lifetime [2], there are many reproductive, dietary and hereditary factors that can contribute to the risk of developing breast cancer.

Non-hereditary factors

Several reproductive factors affect the development of breast cancer. Early onset of menarche increases the risk of breast cancer [3, 4]. Moreover, beginning menstruation at or after the age of 15 reduces the risk by 23% compared to women who began menstruating at age 12. Since early menarche coincides with regular menstrual cycles, girls who begin menstruating early are exposed to hormones that may have a causative effect on breast cancer at an earlier age. Additionally, women with early menarche have increased estrogen levels. Similarly, longer exposure to the premenopausal hormone estrogen, due to later onset of menopause, leads to increased risk of breast cancer [3]. Furthermore, presence of circulating oestrogens and androgens are correlated with increased breast cancer risk in premenopausal women [5]. Bilateral oophorectomies are associated with lower risk of breast cancer compared to natural menopause at the same age. This may be due to the more dramatic decrease in estrogen hormone levels associated with an oophorectomy [3].

The age of a woman's first full-term pregnancy can influence her risk for breast cancer [3]. Pregnancy initially causes a transient increased risk of developing breast cancer for up to 15 years [6-8]. However, in the long-term, childbearing has a protective effect specifically against hormone receptor-positive breast cancer, but has no effect on hormone receptor-negative breast cancer [9]. While it is currently unknown why pregnancy causes a

temporary increase in risk, there are four main hypotheses as to why parity has long-term protective effect: (1) differentiation of breast epithelial cells, causing them to become less prone to transformation (2) changes in responsiveness of breast epithelial cells to estrogen (3) changes in hormone profile, especially the pregnancy hormones estrogen, progesterone, the lactogenic hormone PRL and placental lactogen (4) possible effects of pregnancy hormones on stem cells [6]. Pregnancies that occur before the age 20 cause a 50% reduction in the risk of developing breast cancer, with the first full-term pregnancy continuing to have a protective effect until the age of 35 [6, 10]. However, women who begin having children later in life (over 35) are subject to increased risk of developing breast cancer [11]. This may be because the increased risk that is associated with pregnancy persists for 30-50 years post-partum [12]. The age of subsequent pregnancies has little to no effect on risk development [13]. Therefore, while age of first pregnancy affects risk of developing breast cancer, further studies are needed to fully understand the link between parity and breast cancer.

The same protective effect of parity is observed in rodents. Exposure to pregnancy hormones estrogen and progesterone reduced incidence of carcinogen-induced mammary tumors, and had a protective effect in the p53-null mammary transplant model and the MMTV-Her2 transgenic mouse model [14]. Additionally, studies showed that the basal and progenitor cells of parous mice upregulated differentiation genes while reducing the Wnt/Notch signaling ratio compared to virgin mice. This alteration in signaling led to decreased proliferation potential [15].

In addition to age of pregnancy, other factors associated with childbearing can affect the risk of developing breast cancer. Increased parity of seven or more children decreases the risk of developing breast cancer [16]. Also, a multi-center case control study performed by the Cancer and Steroid Hormone group, compared 4599 women between the ages of 20-55 with confirmed breast cancer to 4536 control women and found that women who

breastfed for over 25 months had a 33% less risk of developing breast cancer compared to women who never breastfed [16]. While it was previously hypothesized that offspring birth size could affect breast cancer mortality, a population-based cohort study found no correlation between child birth size and premenopausal breast cancer mortality or tumor features [17].

Weight and obesity can impact the risk of developing breast cancer. Body mass index (BMI) is often used to define obesity (BMI >30) [18]. Although high BMI is usually associated with increased disease risk, there is an inverse relationship between weight and risk of developing breast cancer in premenopausal women [18]. However, a four year prospective study that included a cohort of 121,964 female registered nurses ages 30-55 demonstrated that while lean women are subjected to increased risk of breast cancer, their tumors tend to be non-metastatic, low-grade tumors [19]. In contrast, overweight or obese postmenopausal women have increased risk of developing breast cancer, with a relative risk of 1.26 [18]. Relative risk is defined as the proportion of an adverse outcome in the intervention group divided by the proportion of adverse outcome in the control group; a relative risk over 1 means that the treatment group is at a higher risk of the poor outcome [20]. Several hypotheses have been proposed as to why obesity increases the risk of breast cancer in postmenopausal women. Adipose tissue is the primary site of estrogen synthesis, where aromatase catalyzes androgens to estrogen. Aromatase levels have a direct relationship with BMI, with a higher BMI increasing exposure to estrogen and estrogen receptor (ER) activation [21, 22]. Moreover, metabolic disease, in which higher amounts of insulin are released into the blood stream due to unabsorbed glucose, can cause activation of IGF-1, a mitogenic factor [21]. Obese women also have increased risk of dying from breast cancer. A prospective study that included participants from the Cancer Prevention Study II followed 495,477 women over a 16-year period demonstrated that grade II overweight women (BMI 35-39.9) had a relative risk of 1.41 and obese women (BMI >40)

had a relative risk of 2.12 of dying from breast cancer compared to lean (BMI 18.5-24.9) women with a relative risk of 1 [21, 23]. Moreover, obese breast cancer patients exhibited higher risk of developing lymph node metastasis, a poor prognostic maker, compared to non-obese breast cancer patients [18]. These findings suggest that the level of obesity directly correlates to risk of dying from breast cancer and aggressive disease. Unfortunately, weight loss upon diagnosis does not appear to benefit the survival of obese women with breast cancer [24].

Hereditary factors

Although most breast cancers are sporadic, 5-7% of breast cancers are due to germ line mutations in either of the breast cancer susceptibility genes *BRCA1* or *BRCA2*. Inheriting just one mutated copy of either gene results in hereditary breast and ovarian cancer (HBOC) syndrome [25]. Women who carry mutations in either gene have a lifetime risk of 60 to 85 percent of developing breast cancer [26]. More specifically, *BRCA1* mutation carriers were found to have 65% and 39% risk of developing breast and ovarian cancer by age 70, respectively. *BRCA2* mutation carriers had a 45% and 11% risk of developing breast and ovarian cancer, respectively. It was found that while the risk for *BRCA1* mutation carriers decreased with age, the same trend was not observed in *BRCA2* mutation carriers [27]. Also, women with HBOC are more likely to be diagnosed with aggressive high grade, triple-negative tumors at an early age [25, 28]. The Ashkenazi Jewish population have a high incidence of about 2% of three specific mutations: BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT [29].

Both proteins participate in the DNA double strand break (DSB) repair pathway homologous recombination (HR) and maintain genomic stability, but have separate functions [30]. During HR, BRCA1 acts upstream of BRCA2 by recruiting other HR proteins to the site of DNA DSB damage and facilitating strand resection. In response to DNA damage, the BRCA1-BARD1 complex can also induce transcription of p21, activating the

G1/S checkpoint, and also has a lesser defined role during the G2/M checkpoint [25]. BRCA2 binds to the HR protein Rad51, mediating its recruitment to the site of the DNA DSB [31]. Without either BRCA1 or BRCA2, cells are forced to undergo a more error-prone form of DNA DSB called non-homologous end joining (NHEJ), potentially increasing genomic instability [25].

Mutation carriers for the *PALB2* gene, which also participates in DNA repair, also have an increased risk of developing breast cancer. PALB2 interacts with both BRCA1 and BRCA2. At 50 years of age, *PALB2* mutation carriers have a 14% likelihood of developing breast cancer, which increases to 35% by age 70 [32]. The association of mutations in PALB2 and other DNA repair proteins and their role in cancer will be discussed in proceeding sections.

1.2 CELL CYCLE

A detailed understanding of normal cell function is required before examining the causes and deregulated pathways associated with cancer. In order to grow and divide, cells must successfully progress through the cell cycle. During the cell cycle, cells must transition through a highly regulated series of orchestrated events that is managed by cyclins, their cyclin dependent kinase (CDK) counterparts and CDK inhibitors (CKIs), leading to the generation of daughter cells (Figure 1, Table 1) [33]. There are two stages of the cell cycle: (1) interphase, which occurs between cell divisions and where DNA is synthesized, and (2) mitosis (M), where the cell divides [34]. If the cell is not undergoing DNA synthesis and division, it can become quiescent and enter a G0 stage [35]. Interphase is composed of gap 1 (G1), DNA synthesis (S) and gap 2 (G2) (Figure 1). Within interphase there are three checkpoints, including G1/S, intra-S and G2/M, which ensure that the DNA is accurately replicated and separated [36]. There are 20 CDKs and four classes of cyclins, including D, E, A and B that form complexes with the CDKs [33]. Cyclin expression oscillates through the cell cycle depending on which CDK activity is needed (Table 1). The CKI INK4 family

includes p16 INK4A, p15 INK4B, p18 INK4C and p19 INK4D, which can selectively inhibit CDK4 and CDK6 activity [37]. The CKI Cip/Kip family includes $p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and can selectively inhibit CDK2 and CDK1 activity [33].

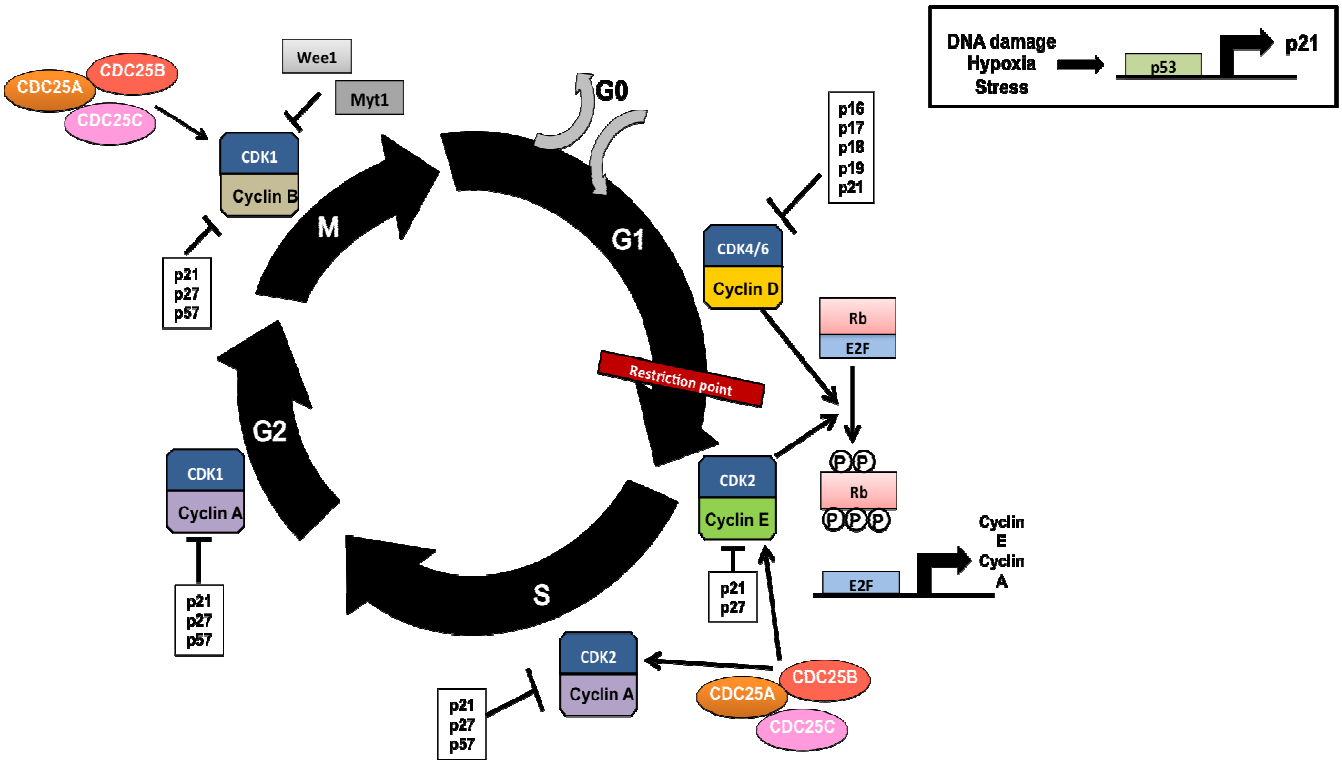


Figure 1: Regulation of the cell cycle. The cell can enter quiescence (G0), or complete G1, S, G2 and M to generate two identical daughter cells. Specific CDK/cyclin activity is required per cell cycle phase. CKIs provide quality control checkpoints to ensure that cells replicate and divide accurately. The transcription factor p53 activates p21 transcription upon cellular stress. During transition through G1, CDK phosphorylation of Rb will release transcription factor E2F to activate S phase genes. Phosphatases CDC25A/B/C remove inhibitory phosphate groups. Kinases Wee1 and Myt add inhibitory phosphate groups to CDK1 upon DNA damage detection.

Table 1. CDK-cyclin complex that regulate the cell cycle			
CDK-cyclin complex	**Peak Activity**	**Substrates**	**Function**
CDK4/CDK6-cyclin D	G1	Rb protein family	Inhibit Rb and release E2F transcription factors
CDK2-cyclin E	G1/S	Rb protein family	Maintains Rb phosphorylation, required for G1-S transition
		p27	Signal for degradation
		Histone 1	Chromosome condensation
		NPAT	Initiate histone gene transcription
CDK2-cyclin A	S	Rb protein family	Maintains Rb inhibition
		Unbound Cdc6	Inhibit re-replication
		DNA polymerase α/δ	Initiate replication
		DNA polymerase d	Elongation
		PCNA	Links CDK2 to DNA replication substrates
		MCM4, 6, 7	Inhibit helicase activity
		Histone 1	Chromosome condensation
		CDC45	Initiate DNA replication, DNA unwinding
CDK1-cyclin A	G2/M	Rb protein family	Required for G2 to M transition
CDK1-cyclin B	M	CDC25C	Positive feedback loop required for continued CDK1 activation
		Histone1/Histone 3	Chromosome condensation
		Nuclear lamins	Nuclear envelope breakdown
		Kinesin-related motors (ex. Eg5)	Centrosome separation
		Microtubule-binding proteins (eg. stathmin)	Spindle assembly
		Condensins	Chromosome condensation
		Golgi matrix components	Golgi fragmentation
		APC E3 ligase	Activate anaphase
CDK7-cyclin H	All	CDK-cyclin complexes	CAK, activate CDKs
Adapted from: [33, 38-40]			

Mitogenic signaling can stimulate quiescent cells to exit GO and enter G1. Mitogenic signaling, including growth factors that can activate the Ras/Raf/Mek pathway, is only required to progress through the first two-thirds of G1 [41, 42]. A major regulator of G1, the retinoblastoma (Rb) protein family consists of Rb, p107 and p130. Together they are known as pocket proteins, due to their conserved binding pocket domain that binds E2F and other oncoproteins [41]. Rb is a tumor suppressor and serves as the molecular determinant of whether the cell will pass through the G1/S transition, known as the restriction point [41]. Once the cell has passed through the restriction point, mitogenic signaling is no longer required as the cell is then committed to completing G1 and transitioning through S phase [43]. Passage through the restriction point occurs 2-3 hours prior to S phase [44]. Rb is maintained in a hypophosphorylated state to inhibit inaccurate progression through G1 [45]. The hypophosphorylated form of Rb binds to the transactivation domains of E2F transcription factor family members, inhibiting their activity. Rb can also inhibit transcription of S phase genes by binding to histone deacetylase (HDAC) [45]. Furthermore, the Rb/E2F complex can act as a transcription repressor complex by binding to the promoters of E2F regulated genes [46]. Upon mitogenic signaling, Ras will activate cyclin D transcription and CDK4 and CDK6 are released from CKI INK4 proteins, allowing them to bind to D-type cyclins (D1, D2 and D3). The CDK4/CDK6- cyclin D complex is shuttled to the nucleus where they are phosphorylated by a CDK activating kinase (CAK). CAK is composed of the CDK7/cyclin H complex (Table 1). Upon CAK activation, the cyclin D-CDK4/CDK6 complex can phosphorylate Rb, forcing it to release the E2F family of transcription factors [37, 41, 47]. E2F target genes include cyclin E- and cyclin-A type cyclins [33]. Cyclin D-CDK4/CDK6 complexes can also bind to Cip/Kip inhibitors, separating them from CDK2 [37]. As the cell progresses through G1, E-type and A-type cyclins are synthesized, with their expression climaxing at the G1/S transition. Cyclin E and cyclin A will associate with CDK2,

further phosphorylating Rb and initiating S phase. Rb has 16 CDK phosphorylation sites [38], where complexes CDK2-cyclin A, CDK1-cyclin A and CDK1-cyclin B1 maintain Rb phosphorylation at specific sites as the cell transitions through the cell cycle [37].

Due to the complexity of DNA synthesis and the vast amount of genetic information that must be replicated, there are an array of errors that can occur during S-phase. Moreover, the cell can be assaulted by genotoxic stress such as ionizing or UV radiation outside of S-phase. Fortunately, the cell has developed three checkpoints, including the G1/S, intra-S and G2/M, to ensure its DNA has been accurately duplicated prior to dividing [36]. While these checkpoints act only at certain times, the proteins used to detect and repair the DNA damage overlap. The detection and repair of DNA damage will be discussed in proceeding sections.

The replication or the G1/S checkpoint prevents progression through S-phase when there is a stalled replication fork due to lack of deoxyribonucleotides, inhibition of the DNA polymerases or the replication fork encountering a damaged piece of DNA [48]. The phosphotyrosine phosphatases CDC25A, CDC25B and CDC25C promote the G1/S transition when they are in their active, unphosphorylated state (Figure 1) [36]. Unphosphorylated CDC25A is an activator of CDK2 and removes inhibitory phospho-groups from CDK2 [49]. Upon detection of DNA damage, either ataxia telangiectasia (ATM) for DNA DSBs or Rad3 related (ATR) for DNA single strand breaks (SSBs) activate Chk2 or Chk1 via phosphorylation, respectively. ATM and ATR are phosphoinositide 3-kinase related kinases (PIKKs). Once activated, Chk1 or Chk2 will inhibit CDK2 activation by phosphorylating CDC25A, causing it to be exported from the nucleus and undergo ubiquitin-mediated proteolytic degradation, inducing a G1 arrest [36]. Activated Chk1 and Chk2 will also phosphorylate downstream proteins that can lead to cell apoptosis (p53), transcription of DNA repair genes (BRCA1, E2F1) and chromatin remodeling (Tlk 1/2) [50].

The tumor suppressor protein p53 can also initiate a G1 arrest. Upon detection of DNA damage, ATM/Chk2 or ATR/Chk1 act together to phosphorylate and stabilize p53 through BRCA1 [36]. BRCA1 forms a complex with BRCA1-associated RING domain protein 1 (BARD1), which must be phosphorylated by either ATM or ATR to aid in the phosphorylation and activation of p53 [51]. p53 has several phosphorylation sites, including serines 15 and 20. Phosphorylation of p53 disassociates it from its negative regulators MDM2 and MDM4, stabilizing its expression [52]. Stabilized p53 activates transcription of p21, which will inhibit CDK2 activity [36]. Activation of the G1 checkpoint should allow the cell to repair any DNA damage prior to progressing into S-phase.

Once the cell transitions through the restriction point, the entire cell genome must be truthfully replicated during S-phase. Preceding S phase, DNA replication begins with the assembly of the pre-replicative complex (pre-RC) on the replication origins of genomic DNA. The first six proteins that bind form a complex called the origin recognition complex (ORC). While multiple replication sites are bound at the same time throughout the genome, not all will initiate DNA replication, with some serving as back up origins. After ORC has been assembled, Cdc6 and Cdt1 are recruited to the origination site, followed by the binding of Mcm2-Mcm7, which serve as a helicase complex that unwinds the DNA [53].

As cells transition into S-phase, CDK2-cyclin A and Dbf4 dependent kinase activity are required for the transition of the pre-RCs to replication forks [33, 53, 54]. At this point, helicase activity unwinds the DNA, the DNA single strands are stabilized and DNA polymerases are loaded onto the DNA. CDK2 phosphorylates Cdc45, allowing it to bind to the Mcm2-7 complex, which is required for DNA unwinding and for binding of the DNA polymerase [53, 54]. The resulting single strands of DNA are stabilized by replication protein A (RPA) binding [55]. Three types of polymerases (pol), including DNA pol α, DNA pol δ and DNA pol ε, participate in the elongation of the newly synthesized DNA. The proliferating cell nuclear antigen (PCNA) protein serves as a clamp for DNA pol δ and ε, linking the

polymerase to the DNA template [56]. Since DNA can only by synthesized 5' to 3', there is a leading strand and a lagging strand. The leading strand is synthesized continuously, while the lagging strand is synthesized in discontinued Okazaki fragments, with the gaps later filled and ligated together. DNA pol α synthesizes RNA primers for the leading and lagging strands, acting as template for the other polymerases. DNA pol α is the only polymerase that that can begin DNA synthesis without primers. DNA pol δ is required for synthesis of both strands [57].

Genotoxic stress can also occur during DNA replication, activating the intra-s-phase checkpoint [48]. This checkpoint acts to temporarily inhibit the firing of DNA origins of replication [49]. Two main pathways work to initiate the intra-S-phase checkpoint upon DNA damage. Similar to the G1 checkpoint, one pathway relies on inhibition and degradation of CDC25A. When activation of ATM/ATR pathway causes CDC25A degradation, CDK2 activity is inhibited, preventing the loading of CDC45 onto the chromatin. Since CDC45 is required for the recruitment of DNA polymerase α, inhibition of CDC25A and CDK2 prevents the firing of new origins [49]. The second pathway acts through ATM-Chk2 to phosphorylate proteins NBS1 and SMC1 to both detect DNA damage and initiate arrest [36, 49].

Before cells can pass through mitosis and divide into two daughter cells, they must pass the G2/M checkpoint. This checkpoint is activated in the event that remnants of DNA damage that were not repaired in G1 or S are detected or DNA damage occurs during G2 [49]. CDK1, the major regulator of mitosis, is inhibited if the cell detects DNA damage through either the ATM/Chk2 or ATR/Chk1 pathways. Wee1 and Myt1 add inhibitory phospho-groups on CDK1 at tyrosine 15 and tyrosine 14, respectively, with CDC25 family removing these phospho-groups to activate CDK1 [58, 59]. If DNA damage is detected, phosphatase CDC25 is inhibited via the ATM/ATR pathways, inhibiting CDK1 activity [59]. The kinase p38 can also inhibit CDK1 activity by sequestering and inhibiting CDC25A. Expression of p53 is required for the maintenance of the G2 checkpoint [49].

The p53 transcriptional targets, p21, GADD45 and 14-3-3σ, act to inhibit CDK1 activity. CDK1 is inhibited by p21 directly, while 14-3-3σ sequesters CDC25 to the cytoplasm, preventing it from activating CDK1. GADD45 separates cyclin B1 from CDK1, also inhibiting CDK1 activity. On its own, p53 can suppress *cyclinB1* and *CDC2* (CDK1) transcription [58].

During G2, in which the cells continue to grow and make proteins in preparation for mitosis, the CDK1-cyclin B1 complex is exported from the nucleus to the cytoplasm. However as cells begin to transition into mitosis, CDK1 or polo like kinase 1 (Plk1) phosphorylate cyclin B1, preventing the complex from being exported. This allows the CDK1-cyclin B1 complex to aggregate in the nucleus and induce mitosis [60]. In addition to CDC25B and CDC25C phosphatase activity, CAK activates and phosphorylates CDK1 at threonine 161 [58]. The five phases of mitosis are prophase, prometaphase, metaphase, anaphase and telophase followed by cytokinesis [39]. The CDK1-cyclin A complex is active during prophase and will facilitate chromatin DNA coiling tightly into two sister chromatids, which requires several post-translational modifications to histones [39, 61]. Also, the centrosomes, which will later pull the sister chromatids apart, migrate to opposite poles. CDK1-cyclin B1 activity is required for the recruitment of motor proteins that will participate in the separation of the two centrosomes. The centrosomes will then begin to organize and nucleate microtubule and promote spindle formation. Also, the CDK1-cyclin B1 complex will facilitate nuclear envelope break down by phosphorylating the nuclear lamin that would otherwise stabilize the nuclear envelope. During prometaphase, the microtubules will attach to the kinetochores located at the centromeres of the sister chromatids. During kinetochore attachment, the cells will proceed to metaphase, where the chromosomes will align at the metaphase plate [39]. The spindle assembly checkpoint prevents entry into anaphase and activation of the anaphase-promoting complex (APC) until there is bipolar attachment of mitotic spindle to each sister chromatid and tension is created between the sisters. As the

cells approach anaphase, where the sister chromatids are separated and pulled towards opposite poles of the cell, APC activity increases. The APC, a multisubunit E3 ubiquitin-protein ligase, is responsible for adding ubiquitin chains to its substrates, targeting them for degradation. The APC has two activators, including Cdc20 and Cdh1, which have differing substrates. APC^{Cdc20} targets both securin, the inhibitor of separase, and cyclin B for degradation [61]. After metaphase, CDK activity is no longer required, and the degradation of cyclin B due to APC^{Cdc20} ubiquitination turns off CDK activity [60, 61]. Also during anaphase, certain CDK1-cyclin A/cyclin B1 substrates must be dephosphorylated. The CDK substrate separase, which is responsible for cleaving the cohesin complex that holds the two sister chromatids together, is dephosphorylated and activated. Additionally, inner centromere protein, the regulatory subunit of Aurora B, must be dephosphorylated to allow Aurora B to migrate to the spindle midzone and encourage spindle stability. APC^{Cdh1} ubiquinates Cdc20 and Plk1 in late anaphase and Aurora kinases A and B in late telophase [61]. As the sister chromatids are pulled apart, the spindle poles separate further. When the chromosomes have reached the poles, telophase is initiated, which includes nuclear envelope reformation and chromosome decondensation [39].

Cytokinesis, or the division of the cytoplasm, is the final step in formation of the two daughter cells. Ingression of the cleavage furrow, the site at the cell equator where the parent cell will contract, begins in telophase. The spindle midzone, or the area of overlapping centrosomal microtubules, will indicate where the cleavage furrow will form. The small GTPase Rho localizes at and contributes to the cleavage furrow by managing actin, myosin II and other actin binding proteins. Kinases Aurora B and Plk1 are required for RhoA recruitment and activity at the cleavage furrow [62]. The microtubules will relay spatial signals to the cytoskeleton at the cell membrane, or the cell cortex [63]. As cytokinesis progresses, the gathering of actin and myosin II at the cell equator pulls together like “purse strings” at the cleavage furrow to form a contractile ring [62]. Once the cleavage

furrow has completely ingressed, an intracellular bridge with a microtubule midbody forms between the two daughter cells [63]. The proteins KIF14 and Citron kinase promote abscission of the two daughter cells followed by membrane fusion [62].

1.3 DEREGULATION OF THE CELL CYCLE IN CANCER

One of the hallmarks of cancer is evasion of anti-proliferation signals. As previously described, normal cells have the ability to arrest or undergo apoptosis if there is damaged DNA. However, tumor cells develop the ability to evade cell cycle checkpoints and continue to replicate through several mechanisms, including increased expression of cyclin/CDK complexes and mutated CKIs (Table 2) [64]. The avoidance of any of the three checkpoints (G1/S, inrtra-S or G2/M) can lead to chromosomal and genomic instability, contributing to tumor progression. Additionally, tumor cells can undergo unscheduled proliferation, in which they will enter the cell cycle without receiving mitogenic signals [65]. This section will discuss the mechanisms that allow tumor cells to proliferate continuously.

Table 2. Failure in cell cycle regulation and cancer		
Gene/ Protein	**Mutation/ Defect**	**Cancer Association**
CCND1	Amplification	HNSCC, NSCLC, endometrial, melanoma, breast and pancreatic cancer
Cyclin D1	Overexpression	HNSCC, NSCLC, endometrial, melanoma, breast, pancreatic and colorectal cancer
	Increased nuclear localization	Lymphoma and prostate cancer
CCNE1	Amplification	NSCLC, lymphoma, breast and ovarian cancer
Cyclin E	Protein truncation/ cytoplasmic localization	Breast and ovarian cancer
Cyclin B1	Increased nuclear localization	Breast and oseophageal cancer
	Overexpression	Breast, colon, prostate, oral, lung and oseophageal cancer
CDK4	Polymorphism R24C	Familial melanoma
CDK4	Amplification	Sporadic melanoma, glioblastoma, osteosarcoma, breast and cervical cancer
Cdc2	Amplification	Colon cancer
CDK2	Amplification	Colon cancer
$p16^{INKA}$	Deletion, point mutation, hypermethylation	NSCLC, glioma, T-cell ALL, mesothiloma, bilary tract, pancreatic and bladder cancer
$p14^{ARF}$	Deletions	Breast, brain and lung cancer
p53	Point and missense mutations	Li-Fraumeni syndrome, colon, breast, lung, brain, pancreas and stomach cancer
	Viral HPV E6 protein	Cervical cancer
Mdm2	Amplification	Leukemia, lymphoma, sarcoma, glioma and breast cancer
Rb	Mutation, loss of function	Retinoblastoma, NSCLC
	Viral HPV E7 oncoprotein	Squamous cell carcinoma and cervical cancer
ATM	Missense, nonsense and truncations	(Ataxia-telangiectasia) Thymic lymphoma, leukemia & breast cancer
ATR	Truncation or missense mutations	Stomach, breast, endometrial
CHK1	Frameshift mutation	Colorectal, gastric, endometrial & small cell lung cancer
CHK2	Truncation or missense mutation	Li-Fraumeni syndrome, breast, bladder, colon, ovary, prostate and lung cancer
AURKA	Amplification	Breast, colorectal and bladder cancer
Adapted from: [37, 65-69]		

CDK-cyclin complexes are often deregulated in cancer. In melanoma, CDK4 often has an R24C mutation that inhibits binding of CKIs, while a variety of cancers have CDK4 and CDK6 gene amplification. Increased CDK4/CDK6 activity can cause hyperphosphorylation of Rb, allowing tumor cells to bypass the G1 checkpoint [37, 65]. Cancers such as leukemia, lymphoma, gastric, colorectal, lung, kidney and breast can have overexpression of cyclin D1 via gene amplification, rearrangement or translocation [37]. This will also increase activity of CDK4/CDK6. Mouse mammary tumorgenesis induced by oncogenes *Ras* and *Neu* requires cyclin D1 expression, and up to 70% of breast cancers show overexpression of cyclin D1 [66, 70]. While E-type and A-type cyclins are often overexpressed and increase activity of CDK2, the *CDK2* gene is not typically mutated in cancer [37, 65]. Additionally, about 6% of cancers have lost function of the F-box and WD repeat domain 7 (FBXW7) protein, which when present ubiquitinates cyclin E and signals for proteasome degradation. However, lack of FBXW7 causes stabilization of cyclin E expression in the nucleus. Aberrant enrichment of nuclear cyclin E can cause chromosomal instability and deregulate the loading of DNA licensing proteins onto the ORCs [71, 72]. CDK1 can have augmented activity in cancers including, breast, colon, lung and prostate, due to overexpression of cyclin B1.

CDK-cyclin complexes can also be mislocalized. Mislocalization of CDK-cyclin complexes at inappropriate times in the cell cycle can cause early or continuous phosphorylation of substrates. Under normal conditions, cyclin D1 is exported to the cytoplasm at the start of S-phase due to glycogen synthase kinase 3β (GSK3β) phosphorylation, which reveals a nuclear export signal. However, a proportion of prostate and lymphoma tumor cells show increased cyclin D1 nuclear localization. Increased cyclin D-1 nuclear localization has been attributed to GSK3β inhibition by increased KRAS or PI3K activity, mutations in the cyclin D1 GSK3β phosphorylation site and mutations in the cyclin-

D1 ubiquitination domain. Consequences of cyclin D1 mislocalization include increased CDK4 activity, chromosome instability and aneuploidy due to DNA re-replication [71]. Cyclin E can be post-translationally cleaved by the serine protease neutrophil elastase to generate oncogenic forms of cyclin E called low molecular weight cyclin E (LMW-E) [73]. Due to a lack of nuclear localization signal, LMW-E is primarily observed in the cytoplasm and has not been detected in normal tissue [74-76]. LMW-E has increased binding affinity to CDK2, is resistant to CDK inhibition by CKIs, causes aneuploidy and accelerates entrance and exit from mitosis, leading to failed cytokinesis and multinucleated cells [77-79]. LMW-E also correlates with poor prognosis in breast and ovarian cancer [80, 81]. Additionally, cyclin B1 can have aberrant localization. During G2/M, cyclin B1 translocates to the nucleus just prior to nuclear envelope breakdown to bind to CDK1. However, cyclin B1 is detected in the nuclei in a high proportion of tumor cells, suggesting early entry into the nucleus. Aberrant localization of cyclin B1 in the nucleus is a poor prognostic marker for breast and oesophageal cancer [71].

Tumor cells can bypass checkpoints by silencing or downregulating CKIs and other endogenous cell cycle inhibitors. The *p16* gene is often subjected to deletion or epigenetic silencing via hypermethylation of its promoter in many cancers, including pancreatic, melanoma, lung, breast and colorectal [35, 67, 82]. Rb can also be mutated or deleted. Inhibition of the Rb pathways generally only requires one “hit,” with manipulations of cyclin D/CDK4, p16 loss or Rb loss being mutually exclusive. To induce cell cycle arrest or cell death, cells with a deregulated Rb pathway may compensate with increased activity of p53. Upstream of p53, ATR can have truncation or missense mutations in stomach, breast and endometrial cancers, inhibiting activation of the DNA damage checkpoint. Additionally, DNA damage checkpoint proteins Chk1 and Chk2 can be deleted or mutated, leading to increased genomic instability and oncogenesis [37].

Over 50% of cancers carry *p53* gene mutations, a major regulator of cell cycle arrest and apoptosis [67]. Most p53 mutations are missense mutations. Some mutations will cause loss of wild-type function or can generate dominant-negative mutants that inhibit wild-type p53 protein [83]. The transactivation capacity of p53 is often lost in cancers such as colon, breast, lung, brain, pancreas and stomach due to alterations in its DNA binding domain [83, 84]. Tumor cells can also inhibit p53 by increasing expression of proteins that inhibit its activity, such as MDM2. Under normal conditions, MDM2 binds to p53 to inhibit its transactivation domain, exporting it from the nucleus for degradation and serves as a p53 ubiquitin ligase [85]. Concordantly, the *mdm2* gene is often amplified in sarcomas and gliomas [86, 87]. Also, deletions in the $p14^{ARF}$ gene, a negative regulator of MDM2, can be found in breast, brain and lung cancers [84]. Viral oncoproteins such as hepatitis B virus X protein and human papilloma virus E6 proteins can bind and inhibit p53 function [88, 89]. Not all p53 mutations are inactivating, with some mutations causing gain of function (GOF) oncogenic activity. GOF Mutant p53 typically has a longer half-life than wildtype p53 and can have changes at the transactivation domain that, while still retaining its DNA binding ability, may cause it to recognize different sequences [90, 91]. Mutant p53 has been shown to affect expression of genes such as *c-myc*, *cyclin D3*, epidermal growth factor receptor (*EGFR*) and others [91]. GOF Mutant p53 can bind to some of the same transcription factors as the wildtype, including E2F1, but alter or increase their activity to promote oncogenicity [90, 92]. Similar to wildtype p53, GOF mutant p53 can also interact with the transcription factor NF-Y. However upon DNA damage, the mutant p53/NF-Y complex activates transcription of genes such as cyclin A, cyclin B2, CDK1 and cdc25C. This is the opposite response of the wildtype p53/NF-Y complex after DNA damage [93]. These examples demonstrate how loss of function and gain of function p53 mutations have a large impact on tumor cells and cell-cycle regulation.

1.4 DNA DAMAGE REPAIR PATWAYS

To protect the genome, the cell has developed several mechanisms to detect, remove and repair DNA damage (Table 3). The cell can incur DNA damage from endogenous sources such as stalled replication forks during DNA replication or exogenous sources such as UV radiation, ionizing radiation or DNA damage agents (i.e. chemotherapeutics). In response to DNA damage, a normal cell will arrest and attempt to repair the damage. If repair is impossible, the cell will undergo cell death [49, 50]. The DNA damage and repair pathway includes damage detectors, signal transducers and downstream effectors [94].

Table 3. DNA Damage and Repair				
	Damaging Agent	**Lesion**	**Repair Pathway**	**Crucial Proteins**
SSB	ROS, adducts, IR and alkylating agents	Altered base	BER	DNA glycosylase, APE1 endonuclease, PARP, DNA polymerase β, DNA polymerase ε, Ligase3, XRCC1, PCNA, FEN1
	ROS, UV radiation, chemical carcinogens	Intra-strand crosslink (bulky adducts)	NER	XPC, XPA, RPA, TFIIH centrin1, XPG, PCNA, EXRCC1-XPF, DNA polymerase δ/ ε, RPA, RFC, CSA, CSB, ligase
	Replication Error	Base mismatch, insertions, deletions	MMR	MSH2, MSH3, MSH6, MLH1-PMS2, EXO1, RPA, PCNA, DNA polymerase δ, RFC, ligase
DSB	Replication error, IR, chemical agents	DSB	NHEJ	Ku70, Ku86, DNA-PKcs, Artemis nuclease, DNA polymerase-μ, DNA ligase IV, XRCC4
	Replication error, ROS, chemical agents, alkylating agents	DSB	HR	Mre11 nuclease, CtIP, Exo1, BLM, DNA2, CDK1, BRCA1, BRCA2 RPA, RAD51, RAD52, RAD54, PALB2, DNA polymerase
ICL	Lipid peroxidation, chemotherapeutics: derivatives of nitrogen mustards & platinum-based	ICL	NER, HR, or Fanconi Anemia Pathway	(NER and HR proteins) FANCM, FA core complex, FAPP24, FANCD2, FANCI, BTR complex
Adapted from: [36, 95-97]				

1.4A SINGLE STRAND BREAKS

Before the cell can repair DNA damage, it must be able detect it and arrest cell cycle progression. The stalling of DNA polymerases during S phase, reactive oxidative species (ROS) and UV radiation can all generate SSBs [36, 48]. During SSB recognition, the single strand DNA will become coated by RPA, recruiting ATR and its regulatory subunit ATR-interacting protein (ATRIP). RPA will also recruit Rad17, which will load the ring structure complex 9-1-1, consisting of Rad9, Hus 1 and Rad1. ATR will phosphorylate Rad17 and 911. ATR will also phosphorylate topoisomerase II binding protein 1 (TopBP1), which is required for ATR activation and Chk1 checkpoint activation [36, 48, 98].

The type of DNA damage determines the mechanism of repair. The three types of repair for SSBs are base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). Cells rely on BER in response to an aberrant base, ROS, chemicals that bind to DNA (adducts), ionizing radiation, and failure in topoisomerase I activity or alkylating agents. A glycosylase enzyme usually recognizes the damaged base and, combined with the endonuclease APE1, facilitates base removal [36, 95]. The type of glycosylase used is dependent upon the substrate that needs to be excised. For example, the uracil DNA glcosylase is required to excise aberrant inclusion of uracil into the DNA, whereas DNA glycosylase SMUG1 excises uracil and oxidized pyrimidines [99]. This causes a gap in the strand that is “cleaned” by poly (ADP-ribose) polymerase (PARP) and polynucleotide kinase to hasten repair [99]. PARP transfers ADP-ribose to form polymer chains. A single nucleotide gap is filled using short-patch repair, while wider gaps require long-patch repair. DNA synthesis that occurs during repair is called repair synthesis. Short-patch repair relies on DNA pol β to add the required nucleotide, followed by ligation by the Ligase3/XRCC1 complex [36]. XRCC1 also serves as a scaffold to recruit other SSB repair proteins. Long-patch repair requires DNA pol β, DNA poly ε and PCNA for repair synthesis.

PCNA helps recruit the endonuclease FEN-1, which is required to remove the 5' blocking group that is displaced as a flap. Finally, ligase 1 seals the DNA backbone [99].

NER serves to repair bulkier DNA damage caused by UV radiation (thymidine dimers), ROS and chemical carcinogens that interrupt the double helix structure [36, 95]. During NER, an oligonucleotide fragment of 25-30 nucleotides is excised [100]. NER acts through two pathways: global-genome NER and transcription-coupled NER (TCNER) [95]. During global genome NER, several proteins, including XPC, XPA and RPA, must first recognize the damaged DNA. XPC is in a complex with HHRAD23A (or HHRAD23B) and centrin2. HHRAD23A may not be required for NER, but aids in NER efficiency, while centrin2 stabilizes binding between HHRAD23A and XPC. Following XPC binding, XPA and RPA bind to the damaged area. Due to the extent of damage, some of the double helix unwinds, facilitating RPA binding to a single strand. Following damage recognition, the six-subunit complex called core transcription factor IIH (TFIIH), which is part of the RNA polymerase II transcription systems, binds to the damaged site. TFIIH is comprised of two DNA helicases (XPB and XPD), which unwind the DNA. The unwinding of the DNA creates a bubble. The proteins p62, p44, p34 and p52 also make up TFIIH and promote bubble formation. Bubble development creates junctions between the unraveled single strand and the remaining helix, which are crucial for accurate excision of the damaged bases. The 3' end of the damaged DNA is cut by XPG, and the 5' end is cut by the EXRCC1-XPF heterodimer. Similar to BER, repair synthesis requires PCNA along with DNA polymerase δ or ε, RPA and replication factor C (RFC), followed by ligase activity to seal the DNA with covalent bonds [100]. The mechanism of TCNER, or NER that occurs during transcription, is not as defined as global-genome NER. Since XPC knockout mice can still perform TCNER, is it is suggested that damaged bases are recognized due to stalled RNA polymerase II activity. The proteins CSA and CSB are required for TCNER, however, their

exact function is unknown. The events of TCNER following damage recognition require TFIIH and are thought to be very similar to global NER [100].

MMR, an intricate process, occurs in response to mismatched bases and insertion or deletion loops formed by DNA replication errors [95]. Mismatched bases are due to DNA polymerase errors that are not detected during proofreading, while insertion/deletion loops (IDL) are due to heteroduplex DNA formation. Heteroduplexes form when two homologous strands from different sources, such as a primer and a template, anneal incorrectly [96]. MMR was first studied in E. coli, where the MutS homodimer was discovered as the error detector. In mammalian cells, the MutS homologs (MSH) MSH2, MSH3 and MSH6 have been found to participate in MMR. The heterodimer MSH2-MSH6 (MutSα) binds and repairs base mismatches and IDLs that are 1-2 nucleotides long, while heterodimer MSH2-MSH3 (MutSβ) repairs base mismatches and IDLs greater than two bases. MutSα and MutSβ interact with the DNA as sliding clamps and can work together [96, 101]. Following binding, MutSα or MutSβ recruits the heterodimer MLH1-PMS2 (MutLα), forming a ternary structure. ATP-hydrolysis causes a conformational change in the MutSα- MutLα complex, allowing it to slide either upsteam or downstream from the damage site. If they slide upstream, they will meet with and displace RFC to allow binding of exonuclease 1 (EXO1) at the 5' end. EXO1 is activated by MutSα and will excise and degrade the damaged strand from 5' to 3', leaving a single strand gap that is stabilized by RPA. Following mismatch removal, MutLα inhibits EXO1. PCNA allows binding of DNA polymerase δ, which synthesizes the new strand, followed by DNA ligase I mediated ligation [96]. In the event that a MutSα- MutLα complex slide downstream towards the 3' terminus, it will meet a PCNA molecule bound at the 3' end of the strand break. EXO1 is also recruited here, degrading the damaged strand. RFC at the 5' end blocks degradation away from the mismatch site in the 5' to 3' direction. Similar to the 5' terminus, EXO1 inactivation is followed by RPA binding, DNA pol δ repair synthesis and DNA ligase I sealing with covalent bonds [96].

1.4B DOUBLE STRAND BREAKS

DNA damage due to collapsed replication forks, ionizing radiation, ROS, alkylating agents and chemotherapeutics can generate DNA DSBs. The two methods to repair DNA DSBs are non-homolgous end joining (NHEJ) and homologous recombination (HR). NHEJ is considered an imperfect, error-prone method of repair since it seals the gaps created by DSB, with a few nucleotides at each end are lost [102]. HR, on the other hand, provides error-free repair because it uses a sister chromatid as a template to fill the gap [103]. Since HR requires a sister chromatid, it is only performed during late S, G2 and M, when a sister chromatid is available [104]. NHEJ can occur during any phase of the cell-cycle [95].

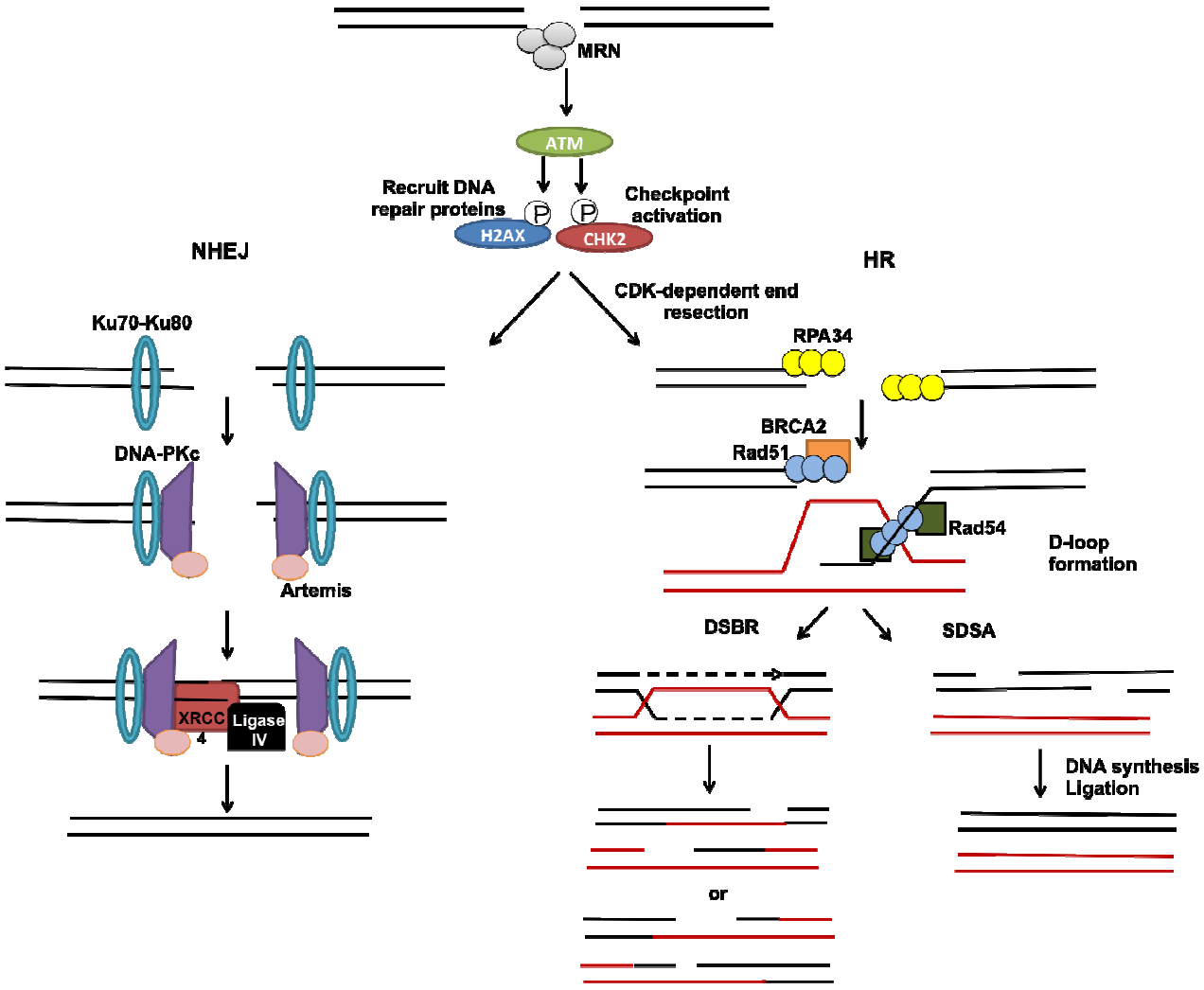


Figure 2. Schematic of DNA DSB repair through NHEJ or HR. In response to DNA double strand breaks, the MRN complex will identify the break and recruit ATM. ATM will phosphorylate CHK2 and H2AX, activating the cell-cycle checkpoint and inducing recruitment of DNA repair foci, respectively. NHEJ can occur at any point of the cell cycle. However, HR is limited to late S, G2 and M because it requires CDK-mediated end resection.

In order for repair to occur, the cell-cycle checkpoint must be activated [105]. The Rad50/ meiotic recombination 11 (Mre11)/ Nijmegen Breakage Syndrome 1 (NBS1) or MRN complex detects and processes DNA DSBs to create single strand DNA (Figure 2) [36, 98, 105]. MRN also serves as a sensor for ATM, with the C-terminal domain of Nbs-1 and the HEAT repeats of ATM mediating the interaction between MRN and ATM [94]. Once recruited to the DNA damage site, ATM will separate from its inactive, dimer form and autophosphorylate itself at S1981, S367 and S1893 [98, 106, 107]. ATM has many substrates, including p53, NBS1, BRCA1 and CHK2 [49, 50]. ATM phosphorylation of Chk2 will activate the cell-cycle checkpoint (Figure 2) [105].

Following MRN formation at the DSB and checkpoint activation by ATM, downstream effectors will be activated to initiate repair. ATM will phosphorylate the C-terminus of histone H2AX at S139 (γ-H2AX), which serves as a major signal of DNA DBS damage and as a recruiter of downstream DNA repair proteins. H2AX can also be phosphorylated by ATR and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Following phosphorylation, the BRCA-1 C-terminus (BRCT) region of the mediator damage checkpoint protein (MDC1) will bind to γ-H2AX by directly binding to the phosphoepitope of H2AX at the C-terminus, enhancing the γ-H2AX signal, and possibly continuing the interaction with ATM [98, 108]. MDC1 also binds to NBS1 of the MRN complex, and is required for the interaction between MRN and γ-H2AX. Moreover, MDC1 and γ-H2AX binding is required for the recruitment of 53BP1, BRCA1, CHK2 and p53, which can also initiate cell arrest or apoptosis [98, 109].

The first repair protein to bind the damage site during NHEJ is the doughnut-shaped heterodimer Ku, composed of Ku70 and Ku86 [110]. Ku will then recruit and activate the PIKK DNA-PKcs, forming the trimeric DNA-PK holoenzyme [95, 102, 104]. During NHEJ, it is imperative that the two damaged ends are kept near each other, in a process called synapsis, to be repaired. It is unclear how exactly this occurs, but it is believed that Ku and

DNA-PKcs play a role [104]. A study using an intermolecular ligation assay reported that Ku could stimulate DNA ligation by bridging the DNA ends while still making them DNA ligase-accessible [111]. Many times the ends of the DNA will be ligated where they, by chance, share 1-4 complementary nucleotides, referred to as microhomology. However, microhomology is not required for end-joining. When microhomology occurs, extra nucleotides beyond the area of microhomology must be trimmed [104]. Ku will also recruit the nuclease Artemis, which is phosphorylated by DNA-PK [110]. Artemis combined with DNA-PKcs, acts as an endonuclease at the 5' and 3' ends [104]. It is the deletion of these nucleotides that can cause a loss of genetic information and an increase in genomic instability [103]. Although polymerase activity is not required in many instances of NHEJ, it has been suggested that the polymerase-μ may fill in some gaps during repair. Following excision of the damaged ends, DNA-PK recruits the DNA Ligase IV/XRCC4 complex to ligate together the blunt ends, completing the process (Figure 2) [104].

As previously stated, HR can only occur during late S, G2 and M phases [104]. To repair DSBs through HR, end resection is performed to form single-stranded DNA. Mre11 nuclease activity combined with CtBP-interacting protein (CtIP) promotes DSB end resection, followed by activity from EXO1, Bloom's syndrome protein (BLM) and DNA2. CDK activity is required for strand resection, as CtIP is phosphorylated by CDK1, limiting HR strand resection to S and G2 (when CDK1 is active) [112, 113]. Ubiquination of CtIP by BRCA1 may also play a role in activating it [114]. MRE11 nuclease activity will stimulate RPA recruitment to coat the 3' ends of the remaining single-stranded DNA. RPA can promote checkpoint induction by binding to ATRIP and activating ATR [103, 115]. RPA must then be replaced with the recombinase Rad51 with the aid of mediators, including BRCA2. While both BRCA1 and BRCA2 have been shown to colocalize with Rad51, BRCA2-Rad51 binding is required for accurate loading of RAD51 at the damage site and completion of homologous recombination [116]. BRCA2 has a DNA binding domain that

can bind both ssDNA and double stranded DNA (dsDNA) and 8 BRC repeats that bind to Rad51 and manage Rad51 filament formation [117]. Rad52 also aids in Rad51 filament formation [105]. Following phosphorylation by Chk2, BRCA1 interacts with Rad51 through BRCA2 and PALB2 [118, 119]. After Rad51 binding, Rad51 and Rad54 enable the resected end of the damaged strand to invade the homologous template, displacing the identical strand and forming a displacement loop (D-loop). Rad51 is disassociated from the single strand by the helicase Srs2 to allow for base complementation [102, 103]. Following D-loop formation, the single strand is extended via DNA synthesis, with the homologous strand serving as the template, causing a D-loop intermediate to form [103]. The intermediate form can be resolved either through synthesis-dependent strand annealing (SDSA) or double strand break repair (DSBR). During SDSA, the invading strand is displaced and re-anneals to the single strand that it was originally bound to in a non-crossover event, allowing no exchange of genetic information. DSBR can be resolved either via crossover or a non-crossover event. During DSBR, the invading strand can be captured at both the ends by the damaged strand, forming Holliday junctions (HJs). Here the HJs can be resolved by either a crossover or a non-crossover event (Figure 2) [120].

1.4C INTERSTRAND CROSSLINKS

Another form of DNA damage that can be lethal for a cell is interstrand crosslinks (ICLs), which are irreversible covalent bonds between bases on opposite strands. ICLs are very damaging because they inhibit the separation of the two DNA strands, which is required for transcription and DNA replication. Chemotherapeutics that are derivatives of nitrogen mustards, such as cycolophosphamide and melphalan, and platinum-based drugs, such as cisplatin, are known to cause ICLs [97]. As a result, there is a risk of patients treated with platinum-based drugs of developing a secondary cancer, especially leukemias [121]. High-fat diets and alcoholism can cause lipid peroxidation that can lead to the formation of ICLs [122, 123]. ICLs left unrepaired can lead to tumorgenesis, and people that

carry germline mutations in the ICL repair pathway are prone to a rare syndrome called Fanconi anemia (FA) [97]. While FA will be discussed in greater detail in proceeding sections, FA cells have provided a source to elucidate ICL repair.

The mechanism of ICL repair is cell cycle dependent. If damage occurs during G1, NER can resect some ICLs. If the ICL damage cannot be repaired via NER, it is carried on into S phase. During S phase the replication fork will stall when it encounters the ICL because the DNA will be unable to unwind [97]. The replication fork collapse can be stabilized through HR, followed by unhooking of the ICL by nucleases MUS81 and EME1, translesion synthesis and then completion through HR [97]. Alternatively, ICL is identified by FANCM [124]. FANCM has an ERCC4 nuclease domain that binds to branched DNA and a domain that interacts with the FA core complex. The FA core complex is composed of 7 FA proteins, including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG and FANCL. Forming a heterodimer with FAAP24, which also has an ERCC4 domain, stabilizes FANCM. FANCM-FAAP24 recruitment of the FA core complex to the site of damage is restricted to cell cycle phases where replication is occurring [97, 124, 125]. The FA core complex includes the ubiquitin ligase FANCL, which monoubiquinates FANCD2 and FANCI [126, 127]. It is believed that monoubiquination of FANCD2 and FANCI stabilizes their binding to the ICL site. Following localization of FANCD2 and FANCI to the ICL site, repair and checkpoint proteins such as RPA (activating ATR), BRCA1, BRCA2 and the Bloom's syndrome complex (BTR) proteins are recruited to the ICL site. BTR is comprised of topoisomerase IIIα, RMI1, RMI2 and BLM, which are required for the resolution of Holliday junctions. ICLs are then resolved through HR mediated by BRCA1 and BRCA2. Unresolved ICLs can lead to cell death, allowing chemotherapeutics that induce ICL to be effective against tumors [97].

1.5 DEREGULATION OF DNA DAMAGE RESPONSE AND CANCER

To become malignant, cancer cells must acquire gene mutations to gain survival and proliferative advantage while avoiding growth arrest or apoptotic signals [64]. Therefore, germline or somatic mutations in DNA damage response (DDR) genes are known to contribute to the development of cancer and impact the effectiveness of cancer treatment (Table 4). Furthermore, epigenetic silencing of DDR genes occurs frequently in cancer. Cells can compensate for the failure of one DNA repair pathway by upregulating another, which can lead to chemotherapy and radiotherapy resistance [128]. The following section will detail which DDR genes are mutated in cancer and how this can affect cancer therapy.

Table 4. Failure in DDR and Cancer				
Repair Pathway	**Protein**	**Mutation/ Defect**	**Contribution to Cancer**	**Ref.**
BER	APE1	High expression	Correlated with drug/radiotherapy resistance in NSCLC & cervical cancer. Poor prognosis in osteosarcoma, medulloblastoma, breast and head and neck cancer	[129, 130]
	XRCC1	Polymorphism R339Q	Decreased efficacy in BER, predisposed to cancer	[131]
	PARP1	Polymorphism V762A	Decreased activity, predisposed to cancer	[132]
NER	XP Proteins	XPA, XPC, XPE, XPF & XPG	Xeroderma pigmentosum: UV sensitivity and skin cancer, especially squamous cell and basal cell carcinoma	[100]
		XPC gene methylation	Correlated to worse outcome in bladder cancer	[133]
		SNPs in XPA & XPC	Correlated to lung and bladder cancer	[134, 135]
		SNPs in XPG (& XRCC1)	Associated with response of NSCLC to platinum-based chemotherapy	[136]
	ERCC1	SNPs	Predictor of response to therapy in lung cancer	[137, 138]
		Gene methylation	Found in glioma, associated with cisplatin sensitivity	[139]
MMR	MSH2, MLH1 & MSH6	Gene promoter methylation	HNPCC (aka Lynch syndrome): early onset (<45) of endometrial, gastric, renal, ovarian and skin cancer.	[140]
DSB repair	NBS1	5 base pair truncation	Nijmegen breakage syndrome (autosomal recessive): early onset of cancer, especially lymphoma (median age 11)	[141]
	MRE11	Point mutations	Associated with ovarian cancer	[142]
		Shortening of poly(T)11 repeat, aberrant splicing	Found in > 90% of colorectal cancers	[143]
NHEJ	Ku70	Polymorphism A46922G	Correlated to breast cancer	[144]
		Epigenetic silencing	Correlated with colorectal, breast & lung cancer	[128]
	Ku80	Gene promoter methylation	Found in > 30% of squamous cell carcinoma	[145]
	XRCC4& Ligase IV	SNPs	Associated with increased risk of glioma	[146]

HR	BRCA1 & BRCA2	Germline mutation of BRCA1	Increased risk of breast (> 70%) and ovarian cancer (>40%) by age 70	[147]
		BRCA1 promoter methylation	Found in spontaneous breast, ovarian & lung cancer	[148]
		Germline mutation of BRCA2	Increased risk of breast, ovarian, colorectal, esophageal, pancreatic, stomach and hematopoietic cancer	[149]
	Rad50	Frameshift mutation, protein truncation	Found in >30% gastrointestinal cancers	[150]
	PALB2	Monoallelic truncations, germline mutations	Increased risk of breast cancer 2 – 4 fold, correlated to pancreatic cancer	[32]
ICL	FANC proteins	FANCA, FANCC, FANCG, FANCF, FANCE, FANCB, FANCD2, & FANCI	High risk of AML (>50% by age 40), HNSCC, oesophageal & gynecological cancer	[151]
		Gene methylation of FANCF	Associated with neck squamous cell carcinomas, NSCLC, cervical & ovarian cancer	[152-154]
		Biallelic mutation FANCD1 (BRCA2)	Increased risk of early onset of childhood tumors, including Wilm's tumor & medulloblastoma (mortality by 5 yrs. of age)	[155, 156]
		FANCN (PALB2)	Fanconi anaemia N: early onset of Wilm's tumor & medulloblastoma	[157]

Several members of the BER pathway are mutated in cancer. The R339Q mutation in XRCC1 can decrease BER efficiency and can increase the risk of developing cancer [131]. Furthermore, a variety of tumors have overexpression of the endonuclease APE1, which can protect against radiotherapy and chemotherapeutics, making it a viable target [129, 130].

Mutations in the XP proteins, including XPA, XPC, XPE, XPF and XPG, of the NER pathway predispose people to the autosomal hereditary disease Xeroderma pigmentosum (XP) [158]. Mutations in XP proteins cause defects in the NER pathway that leads to sensitivity to UV radiation, leading to cell transformation, with the median age of onset for skin cancer at 8 years old [100, 159]. Patients with XP most commonly have basal cell or squamous cell carcinoma, but can also have melanoma, keratoacanthomas, angiomas and sarcomas [100]. XP patients also have a higher incidence of non-skin cancer malignancies, including brain tumors, leukemias and lung cancer [160, 161]. Accordingly, epigenetic silencing via methylation and single nucleotide polymorphisms (SNPs) of *XPC* increases risk of bladder cancer [162]. Also, SNPs in *ERCC1* are associated with skin cancer and poor prognosis in non-small cell lung cancer (NSCLC), further demonstrating the importance of a functional NER pathway [161, 162].

Defects in the MMR pathway, due to promoter methylation of MLH1 or mutations in MSH2 or MSH6, cause the development of autosomal dominant hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome. Lynch syndrome increases the risk of developing colorectal, stomach, ovarian and endometrial cancer [140]. Moreover, defects in the MMR pathway can cause resistance to the chemotherapeutic cisplatin, but are more sensitive to other cross-linking agents [163-165].

The failure to detect DSBs due to a mutations or truncations in *NBS1* causes Nijmegen breakage syndrome. Nijmegen breakage syndrome is characterized by immunodeficiency, ovarian dysgenesis and infertility, chromosome instability, sensitivity to

radiation and high risk of developing cancer, especially lymphomas, at a very young age [141]. Point mutations in Mre11, another member of the MRN complex, are associated with hereditary breast and ovarian cancer [142]. Inactivated and mutated MRE11 is associated with MMR-deficient cancers [143].

There are several mutated NHEJ proteins that contribute to cancer development. SNPs in Ku70 and Ku80 are correlated with the development of breast cancer, while epigenetic silencing of Ku80 correlates with lung cancer and squamous cell carcinoma [128, 145, 166]. SNPs in ligase IV and XRCC4 are associated with increased risk of developing glioma [146]. Due to the importance of NHEJ to repair DSBs, DNA-PKc inhibitors are being developed to radiosensitize cancer cells, particularly in B-cell chronic lymphocytic leukemia [128].

Similar to NHEJ, members are of the HR pathway are often deregulated in tumors, allowing them to maintain a higher level of genomic instability that is characteristic of cancer. Germline mutations in the BRCA1 and BRCA2 genes are most frequently associated with developing HBOC, but are also known to increase the risk of developing prostate, pancreatic, melanoma and gastrointestinal tumors [147, 167, 168]. Moreover, even sporadic breast and ovarian tumors have been shown to have *BRCA1* silencing via promoter methylation [148]. PARP inhibitors are being used to target BRCA-deficient tumors. This is based on the premise that PARP inhibition prevents repair of SSBs, generating DSBs and forcing cancer cells to rely on HR to resolve DNA breaks; BRCA-mutated cells are HR-deficient causing them to undergo cell death [169, 170]. Moreover, inhibiting HR can sensitize tumors to targeted therapy. For example, CDK inhibition can reduce BRCA1 recruitment, sensitizing BRCA1 wildtype tumors to PARP inhibition [171]. Most recently, germline truncations, alternative splicing and deletion mutations in *PALB2* were shown to increase the lifetime risk of developing breast cancer, especially in premenopausal women [32]. Gastrointestinal cancers with microsatellite instability can carry

frameshift mutations in *Rad50* and *BLM* [150]. HR-defective cancers have been shown to be sensitive to DSB-inducing agents [172]. Defective HR is paradoxical in that it allows cancer cells to accumulate the gene mutations necessary for continued proliferation, while providing drugable targets and sensitizing cancer cells to DNA damaging agents.

Failure in ICL repair due to gene mutations in the FA pathway is characteristic of Fanconi anemia, a rare genetic disease that causes decreased fertility, congenital abnormalities, bone marrow failure and increased risk of hematological and squamous cell cancer [173]. FA is diagnosed via cytogenetic analysis in which lymphocytes are subjected to ICL-inducing agents, such as platinum-based chemotherapeutics. FA-positive cells are hypersensitive to ICL-inducing agents, showing increased chromosomal breaks [174]. A majority of FA patients suffer from *FANCA* (65%), *FANCC* (15%) or *FANCG* (10%) mutations. However, mutations have been found in a variety of FA pathway genes, including FANCD2, FANCI and other HR genes [128]. Again, the high prevalence of cancer in carriers of FA gene mutations emphasizes the importance of effective DNA repair pathways.

1.6 MOLECULAR SUBTYPES OF BREAST CANCER

Having detailed some of the major causes and deregulated pathways associated with cancer, this section will describe the molecular characteristics of breast cancer and how they impact treatment strategies. Breast cancer is a heterogeneous disease due to differential gene expression, which affects treatment response and prognosis. Microarray analysis was used to delineate five molecular breast cancer subtypes, including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive, basal-like and normal-like breast cancer (Table 5) [175, 176]. Additionally, a claudin-low subtype and seven distinct subtypes within basal-like breast cancer have been identified [177, 178]. The subtype of breast cancer determines how the tumor responds to targeted therapy or DNA damage inducing treatments, including chemotherapy and radiotherapy. Molecular

subtypes also present differently in the clinic [179]. This section will detail the characteristics of all the molecular subtypes and new promising therapies entering the clinic.

Table 5. Molecular sub-types of breast cancer

	Expression Profile	Prevalence (%)	Prognosis	Treatment options
Normal-like	Lo PARP1 and Chk1, hi ALDH	3-10	Good	Undetermined
Luminal A	ER+, PR+, HER2-, lo ki67	24-39	Good	Endocrine therapy
Luminal B	ER+(low), PR+ (low), HER2+, hi ki67	10-18	Poor	Endocrine therapy plus chemotherapy
HER2 positive	ER-, PR-, HER2+	12-25	Poor	Trastuzumab, lapatinib (plus chemotherapy)
Basal-like	ER-,PR-, HER2-, CK 5/6, 14,17, EGFR	15	Poor	Chemotherapy, surgery
Claudin-low	Lo claudin 3,4,7, occuldin, lo E-cadherin, hi $CD44^{+}/CD24^{-/low}$	12-14	Poor	Chemotherapy (dependent upon hormone/HER2 status)

Adapted from: [175, 176, 178, 180-182], Lo = low, Hi = high, CK = cytokeratins

1.6A NORMAL BREAST-LIKE CANCER

Up to 10% of breast cancers are diagnosed as normal breast-like tumors, which are composed mostly of normal cells, including stromal cells, and show high expression of genes found in adipose tissue [180, 183]. These tumors also have decreased expression of proliferation genes compared to Luminal B, HER-2 and basal-like subtypes, while being enriched for mesenchymal and extracellular matrix genes [184, 185]. Normal breast-like tumors have low expression of PARP1 and Chk1, but have high expression of the stem cell marker ALDH1. ALDH1 expression is also common in stromal cells [185]. Patients with normal-breast like tumors show lower overall survival and recur sooner than patients with the Luminal A subtype [180].

1.6B LUMINAL A

Luminal A breast cancer is more prevalent in post-menopausal women [181]. Both luminal breast cancer sub-types are ER-positive. Immunohistochemistry (IHC) staining is used to detect ER expression; as little as 1% of cells staining positive for ER qualifies the tumor as ER positive [186]. The ligand estradiol binds to ER, activating ER to go to the nucleus where it regulates gene expression [187, 188]. The luminal A subgroup is characterized as being HER2 negative and progesterone receptor (PR) positive, with higher ER expression and lower expression of the proliferation markers Ki-67 and PCNA than luminal B tumors [189-192]. Luminal A tumors also have overexpression of GATA binding protein 3, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3α and estrogen regulated LIV-1 [176, 180]. Luminal A tumors often present as histologically low-grade tumors with good differentiation [191]. Also, a majority of grade 1 luminal A tumors show genomic loss of chromosome 16q, which is correlated with good prognosis [190]. As a result, patients with luminal A tumors have a lower probability of developing distant metastasis, and have increased overall survival compared to patients diagnosed with other subtypes [176].

The treatment breast cancer patients receive is based on the disease stage and pathological features, including receptor status and tumor grade. Patients with luminal A breast cancer are often treated with endocrine therapy, including selective estrogen response modulators (SERMs), such as tamoxifen and raloxifene, which can bind to ER and inhibit its activity in cancer cells [193]. Treatment with tamoxifen is often combined with breast conservation surgery, in which a lumpectomy is performed followed by radiotherapy. As adjuvant therapy, 5 years of tamoxifen reduces risk of recurrence by 47% and death from ER-positive breast cancer by 26% [194]. Tamoxifen and raloxifene are also effective as preventive agents for women who are at high risk of developing invasive cancer. Unfortunately, tamoxifen is associated with increased risk of developing endometrial cancer. However, raloxifene is associated with lower rates of endometrial cancer and can equally prevent the development of invasive cancer in high-risk patients [195]. While SERMs have efficacy in both pre-menoposual and post-menopausal women, post-menopausal women often receive aromastase inhibitors, including anastrozole, letrozole and exemestane [193]. As described previously, aromatase is an enzyme that converts androgen into estrogen [22]. A phase III clinical trial found that anastrozole extended time to progression (TTP) in post-menopausal women with metastatic breast cancer (MBC) to 11.1 months versus 5.6 months with tamoxifen treatment [196]. Moreover, letrozole increased TTP to 41 weeks versus 26 weeks with tamoxifen treatment when used as a first line of therapy in post-menopausal patients with advanced ER positive breast cancer [197]. Notably, the multi-center 10-year Arimidex tamoxifen alone or in combination study found that while anastrozole significantly improved disease-free survival (DFS) and time to recurrence compared to tamoxifen alone or in combination, it did not significantly improve overall survival in post-menopausal women with hormone positive breast cancer [198]. The efficacy of endocrine therapy as neoadjuvant therapy, or therapy given prior to surgery, is still being studied, but initial reports suggest improved response of letrozole or anastrozole compared to tamoxifen [193].

The anti-ER agent fulvestrant is used as a second-line therapy in post-menopasual patients with advanced ER-positive breast cancer, and is effective in tamoxifen refractory MBC disease [199]. Overall, due to the development of endocrine therapy and a non-aggressive disease, patients with luminal A breast cancer have a good prognosis and fare better than patients with other sub-types.

1.6C LUMINAL B

Tumors classified as luminal B have decreased ER positivity, have little or no expression of PR, are more likely to be HER2 positive (20% via IHC and mRNA) and have higher expression of Ki-67 compared to luminal A tumors. Although high expression of Ki-67 has been defined as greater than 13.5% positively stained cells, there have been discrepancies in the scoring of Ki-67 via IHC, and the method is not standardized yet [189, 200, 201]. Microarray data shows increased gene expression of cyclin E, peroxiredoxin, squalene epoxidase, gamma-glutamyl hydrolase and nuclease sensitive element binding protein 1 are all specific to luminal B tumors [176, 180]. Also, about one third of luminal B tumors carry TP53 mutations [202]. Luminal B tumors are distinguished from luminal A tumors as being high grade and showing poor differentiation, with patients having increased risk of recurrence and distant metastasis primarily to the bone and lung. Moreover, use of the OncotypeDX, a diagnostic program that predicts outcome and optimal treatment based on molecular characteristics of the tumors, often gives luminal B breast cancer a high recurrence score (RS). The increased risk of metastasis and overall more aggressive disease results in a decreased overall survival compared to patients with luminal A breast cancer [183, 191, 193, 203].

Luminal B breast cancer patients are less responsive to endocrine therapy, and benefit from the addition of chemotherapy for treatment [201]. Post-menopausal, ER-positive breast cancer patients with high RS were at greater risk of developing distant recurrence when treated with endocrine therapies anastrozole, tamoxifen or in combination

compared to patients with low or intermediate RS [204]. A clinical trial with node-negative, ER-positive patients measuring the risk of distant recurrence in low, intermediate or high RS patients who received tamoxifen or tamoxifen plus the chemotherapies cyclophosphamide, methotrexate and fluorouracil (CMF) or MF found that only high RS patients saw a 28% increase in distant recurrence free survival, versus only a 1.1% benefit in low RS patients [205]. Moreover, a phase III trial with node-positive, ER-positive post-menopausal patients found that 57% of patients that received cyclophosphamide, Adriamycin (doxorubicin) and fluorouracil (CAF) prior to or concurrent with tamoxifen had DFS versus 48% of patients who received tamoxifen alone during a 10-year follow-up [206]. While the CAF cocktail is effective, a trial comparing CAF versus docetaxel, doxorubicin and cyclophosphamide (TAC) found that 85.2% of patients with luminal B breast treated with TAC had 3 years of DFS versus 79% treated with CAF [207, 208]. Therefore, patients with luminal B breast cancer benefit from the combination of endocrine therapy and chemotherapy, but the aggressiveness of the disease still calls for more efficient treatment strategies.

There are novel therapies being developed to treat luminal B breast cancer that would target other pathways involved in proliferation and drug evasion. For example, targeting the mTOR pathway in combination with letrozole has shown to be promising both in neoadjuvant and metastatic settings [209, 210]. Combination of the mTOR inhibitor everolimus with tamoxifen in patients with aromatase inhibitor pre-treated metastatic disease increased progression free survival (PFS) by four months and increased overall survival [211]. In a phase II trial, inhibition of fibroblast growth factor receptor (FGFR) in patients with luminal metastatic disease that had amplification of *FGR1,* a known mechanism for endocrine resistance, induced either a partial or stable response in patients [201]. Overall, new treatment strategies that target pathways known to participate in endocrine resistance appear to be promising for patients with luminal breast cancer.

1.6C HER-2-POSITIVE BREAST CANCER

HER-2, or ERBB2, is a member of the epidermal growth factor (EGF) receptor family, which is a subclass of the receptor tyrosine kinases superfamily. The fours receptors within the EGF family are EGFR/ERBB1, ERBB2 (HER-2), ERBB3 and ERRB4, all of which are transmembrane proteins with extracellular ligand binding domains and cytoplasmic tyrosine kinase domains [212]. All of the EGF receptor family members, except HER-2, have specific ligands that cause receptor activation. HER-2 has no soluble ligand, but is the preferred partner to form heterodimers with other activated EGF receptors [213]. Receptor activation leads to homodimer or heterodimer formation, followed by phosphorylation of the cytoplasmic tail, which acts as docking site for other proteins to activate downstream signaling [212]. Downstream pathways activated by HER-2 signaling include the mitogen-activated protein kinase (MAPK) pathway and the PI3K pathway [214, 215]. HER-2 can also sequester the CDK inhibitor p27, preventing it from inhibiting CDK2 activity, promoting proliferation [216].

HER-2 gene amplification and overexpression, detected through either fluorescence in situ hybridization or IHC, occurs in 12 to 25 percent of breast cancers [183, 217]. HER-2 overexpression has also been detected in ovarian, gastric and salivary tumors [212, 218, 219]. The HER-2 pathway is constitutively active when HER-2 is overexpressed, as it can form homodimers with itself [212]. Patients with HER-2 positive breast cancer have a poor prognosis and decreased overall survival compared to non-HER-2 positive breast patients, with a majority of tumors being poorly differentiated and high-grade [181, 218]. HER-2 positive breast cancer preferentially metastasizes to the liver, brain and bone [203].

However, targeted therapies have been developed to treat HER-2 positive breast cancer. The recombinant, humanized monoclonal antibody trastuzumab (Herceptin) binds to the extracellular domain of HER-2, inhibiting its activity and sequestering it away from other EGF receptors [193]. Since, trastuzumab had an objective response rate (RR) of 35%

in HER-2 overexpressing MBC patients, it was initially only approved as a single agent [220]. However, subsequent trials evaluated the combination of trastuzumab with an anthracycline (either doxorubicin or epirubicin) and cyclophosphamide or paclitaxel (in patients who had previously received anthracycline chemotherapy) in HER-2-positive MBC patients. When comparing trastuzumab plus chemotherapy versus chemotherapy alone, time to disease progression increased to 7.4 months versus 4.6 months, with median survival increasing to 25.1 months versus 20.3 months. Unfortunately, 27% of patients who received an anthracycline with trastuzumab had cardiac dysfunction [221]. As a result, trastuzumab is only used in combination with non-anthracycline chemotherapies [193]. Moreover, trastuzumab has been approved to be included in adjuvant therapy in patients with localized breast cancer, due to a 52% decrease in risk of disease recurrence and 91.4% overall survival compared to 86.6% of the control group with a 24 month follow-up [222]. The small molecule inhibitor lapatinib binds to and inhibits the tyrosine kinase domain of EGFR and HER-2 [193]. The addition of lapatinib to capectiabine versus capecitabine alone increased time to progression to 8.4 months versus 4.4 months in HER-2 positive MBC patients who previously received anthracyclines, taxanes and trastuzumab [223]. A randomized, open-label phase III trial examined lapatinib alone, trastuzumab alone, or in combination in HER-2 positive breast cancer patients, and found that 51.3% of combination treated patients had pathological complete response (pCR) versus only 29.5% of trastuzumab treated patients [224]. Inhibition of EGFR alone via gefitinib, or in combination with trastuzumab, has not been effective in breast cancer patients [193]. While HER-2 positive breast cancer is an aggressive disease, these patients can benefit from targeted therapy as single agents or in combination with chemotherapy.

1.6D BASAL-LIKE BREAST CANCER

About 15% of breast cancers are classified as the aggressive subtype basal-like breast cancer (BLBC). Forty percent of BLBC patients are premenopausal African American

women [181]. BLBC is characterized through gene-expression profiling as having expression of high-molecular weight basal cytokeratin (CK) 5/6, CK14 and CK17 and expression of EGFR, with 15-35% of BLBC tumors having EGFR gene amplification. Frequently, BLBC is ER and PR negative and HER-2 negative/low and have high Ki-67 expression by IHC, characteristics that overlap with triple-negative breast cancer (TNBC) [225, 226]. However, since not all BLBC are hormone receptor and HER-2 negative, BLBC is not synonymous with the clinical diagnosis of TNBC [226]. Up to 82% BLBC have been found to have *TP53* gene mutations [180]. The similarities between sporadic BLBC and breast cancer due to germline mutations in *BRCA1* have become increasingly recognized. A majority of *BRCA1* mutation breast cancers are also negative for hormone receptors and HER-2 and carry *TP53* mutations [28, 227]. BRCA1 mRNA has been found to be lower in BLBC sporadic tumors compared to non-BLBC sporadic tumors [228]. Moreover, both BRCA1 tumors and sporadic BLBC tumors present as high-grade tumors that have high expression of EGFR, Skp2 and cyclin E and low expression of cyclin D1 and p27 expression [28, 229]. Therefore, women carrying *BRCA1* mutations who are at high risk of developing breast cancer are more likely to develop BLBC.

Patients with BLBC have a poor prognosis, with decreased overall survival and decreased relapse-free survival, especially during the initial 3-5 years after diagnosis, compared to the other subtypes [180, 230]. Unlike non-BLBC tumors, BLBC preferentially metastasizes to the brain and lung, with patients dying within 5-8 years of diagnosis [203, 226]. Moreover, the basal-like phenotype predicts aggressive disease and low survival after the development of metastasis [231].

Since BLBC tumors can be ER, HER-2 low or triple-negative, BLBC status does not currently denote a specific type of treatment regimen. Therefore, patients with ER-positive or HER-2 positive BLBC tumors receive endocrine therapy and trastuzumab, respectively. However, most BLBC tumors are triple-negative, which cannot benefit from currently

available targeted therapies, and only receive chemotherapy [226]. Treatment of TNBC will be discussed in greater detail in the following section. Therefore, BLBC is an aggressive disease with high risk of recurrence, and the therapies used to treat this subtype are based on hormonal and HER-2 status.

1.6E CLAUDIN-LOW BREAST CANCER

The claudin-low subtype is a more recently identified subtype using gene-expression analysis of human breast tumors and murine mammary tumors from transgenic mouse models [178]. Claudin-low tumors have low expression of the tight junction proteins claudin 3,4,7 and occuldin and the epithelial marker E-cadherin. Claudin-low tumors share some characteristics of basal-like tumors, including low expression of HER2 and luminal markers [178, 184]. These tumors have high expression of immune response genes, including genes that are typically expressed by B-cell and T-cell lymphocytes. The presence of lypmphocyte proteins indicates immune cell infiltration; however, the expression CXCL2 most likely occurs within the tumor cells [185]. Claudin-low tumors are also enriched for cells that express the tumor initiating stem cell markers $CD44^{+}/CD24^{-/low}$ [232]. Additionally, these tumors have high expression of mesenchymal and stem cell markers N-cadherin and vimentin [184]. Moreover, tumor expression of $CD44^{+}/CD24^{-/low}$ was more enriched in the claudin-low subtype following endocrine and chemotherapy treatment [233].

Only about 12-14% of breast cancers are classified as claudin-low tumors. Claudin-low tumors are predominantly triple negative, with only up 25% being hormone-receptor positive. Patients with claudin-low tumors recurred sooner than the luminal A subtype and have lower overall survival than either luminal A or luminal B [184, 234]. There are currently no specific therapies to treat the claudin-low sub-type. Similar to basal-like tumors, these tumors receive treatment based on their hormone-receptor and HER2 status.

1.7 TRIPLE-NEGATIVE BREAST CANCER

TNBC is a clinical diagnosis given to patients with breast tumors that lack expression of ER, PR or *HER-2* amplification [177]. Up to 10- 20% of breast cancers are TN, with patients predominantly consisting of premenopausal, African American patients. In fact, 20.8% of African American patients with breast cancer are triple-negative, versus only 10.4% of Caucasian patients [235]. TN tumors are aggressive with patients presenting at an advanced stage at time of diagnosis, with large, high-grade tumors that have metastasized to the lymph nodes [236]. Moreover, TNBC patients have a poor prognosis due to an increased rate of distant metastasis within the first five years of diagnosis, peaking at 3 years, compared to patients with other subtypes who remained constant throughout. Less than one third of women with metastatic TNBC survive 5 years [237, 238]. Similar to BLBC, TNBC preferential metastatic sites include, lung (40%), brain (30%), liver (20%) and bone (10%), differing from non-TNBC [239, 240]. As previously described, 70% TNBC tumors are classified as BLBC sub-type, with the remaining tumors falling into other subtypes [241]. TNBC tumors are more likely to occur in women with *BRCA1* gene mutations, with 80% of breast cancer patients with *BRCA1* mutations diagnosed as TN [227, 241, 242]. Over half of TNBC tumors have EGFR overexpression, which is correlated with poor prognosis [243]. Additionally, TNBC tumors frequently have deregulated cell cycle machinery, including high expression of cyclin E, low expression of cyclin D and aberrant expression/ mutations in the tumor suppressor p53 [80, 244-246]. Also, a majority of TNBC tumors have loss of Rb, but this is correlated with a good prognosis [247]. Overall, TNBC tumors differ both molecularly and clinically from non-TNBC tumors (Table 6).

Table 6. Comparison of TNBC and non-TNBC tumors		
	TNBC	**non-TNBC**
Mean age at diagnosis (yrs.)	53	57.7
Age < 40 at diagnosis (%)	12.2	5.7
Tumor size at diagnosis (cm)	3	2.1
Lymph node mets. (%)	54.4	45.6
Grade III tumors (%)	66	28.3
Poorly differentiated (%)	71.1	26.5
Sites of distant metastasis (most likely to least likely)	Lung, brain, liver, bones	Bones, liver, lung, brain
Basal cyotkeratins (%)	71	6.3
EGFR (%)	>60	2-16.5
p53 mutation (%)	54-82	13
Mitotic index	High	Sub-type specific
Rb loss (%)	64.5	6.5-22.6
Adapted from: [237], [240], [245, 247, 248]		

The heterogeneity of TN tumors was illustrated in gene expression analysis that generated 6 subtypes within TNBC, including basal like 1 (BL1), basal like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesecnchymal stem-like (MSL) and luminal androgen receptor (LAR) [177]. To determine if these subtypes could predict response to treatment, breast cancer cell lines used in the laboratory were also subtyped. The BL1 subtype is enriched in cell cycle and cell division genes and the DNA damage ATR/BRCA pathway genes. BL2 tumors have high expression of the EGF, WNT and IGF1R pathway genes. Both BL1 and BL2 tumors have higher Ki-67 expression compared to the other subtypes. Cell lines within the BL1/BL2 classification were much more sensitive to the chemotherapeutic Cisplatin compared to other subtypes. However, not all cell lines responded equally to the PARP inhibitors veliparib and olaparib. IM tumors are enriched for immune response genes, including the IL-12, IL-7, B cell receptor and natural kill cell pathways as well as the TNF and JAK/STAT signaling pathways. Genes involved in cell motility characterize the M subtype, including the Rho pathway, as well as extracellular matrix receptor interaction genes, including WNT, ALK and TGF-β pathways. In addition to Rho, TGF-β and ALK pathways, MSL tumors are enriched for EGFR, PDGF and ERK1/2 pathway genes. Both M and MSL tumors have higher expression of EMT genes, including *MMP2, TWIST1 and ZEB1*. Compared to M tumors, MSL tumors have lower proliferation along with higher expression of stem cell genes. Cell lines within this subtype are more sensitive to dasatinib, an inhibitor of the cell migration protein Src, than the LAR cell lines. Tumors within the LAR subgroup are enriched for androgen and estrogen metabolism, steroid biosynthesis, TCA cycle and sucrose and fatty acid metabolism genes [177]. LAR and a subset of MSL cell lines were sensitive to the AR antagonist bicalutamide, whereas basal-like cell lines had IC_{50} values greater than 500 um [177]. The identification of TNBC tumor subtypes could potentially lead to more personalized care for these patients.

In contrast, a recent paper that employed RNA and DNA profiling analysis on TNBC tumors identified 4 TNBC subtypes, including Luminal-AR (LAR), Mesenchymal (MES), basal-like immune suppressed (BLIS) and basal-like immune activated (BLIA) [249]. The conflicting results between the Lehman et al. study and the Burstein et al. study may be due to the method used to confirm the TN status of tumors analyzed. The LAR subtype is characterized by increased gene expression of cyclin D1, dehydrogenase/reductase member 2, prolactin-induced protein and androgen receptor. Possible druggable targets against the LAR tumors include ER, PR and mucin 1 cell surface receptor. The MES subtype shows high expression of EGFR and alcohol dehydrogenase 1B, with possible druggable targets including neurotrophic tyrosine kinase receptor type 2, endothelin receptor type B and interleukin 1 receptor. The BLIS subtype is characterized by increased expression of fibroblast growth factor receptor 2 (FGFR2) and E74-like factor 5. Patients with BLIS tumors show decreased disease-free survival compared to the other subtypes. Overexpression of FGFR2 provides a potential target for treatment of the BLIS subtype. Tumors in the BLIA subtype have gene amplification of CDK1, chemokine ligand 9 and topoisomerase II alpha. In addition to topoisomerase II, possible drug targets against BLIA are chemokine ligand 10 and proteasome subunit beta type 9. Overall, patients with BLIA tumors have a good prognosis and have increased disease-free survival compared to the other subtypes [249]. Further validation will most likely be required to consolidate the findings of both TNBC subtype studies. In any case, the subtyping of TNBC will hopefully lead to the development of more targeted therapies.

Since TN tumors do not express ER or HER2, they do not respond to current targeted therapies, including tamoxifen and trastuzumab. As a result, TNBC patients receive chemotherapy and surgery for treatment [248]. There is no standard of care for TNBC patients, with patients receiving a variety of chemotherapeutics including anthracyclines, fluropyrimidines, taxanes and platinum-based drugs (Table 7). The

alkyating agent cyclophosphamide, which forms interstrand and intrastrand crosslinks, is often added to anthraycline-based therapy to increase therapeutic benefit [250]. TNBC patients can also receive platinum-based chemotherapy both as a single agent and in combination therapy, with TNBC patients responding better to platinum based therapy compared to non-TNBC patients [251]. TNBC tumors are more likely to have pCR compared to ER positive tumors (22% vs 11%) to neoadjuvant chemotherapy. A study that monitored patients treated with paclitaxel followed by treatment with 5-fluorauracil, doxorubicin and cyclophosphamide (FAC), found that TNBC patients had a higher rate of pCR of 45%, compared to only 6% in luminal tumors [179]. The delivery method of combination treatments can impact treatment efficacy. For example, simultaneous administration of doxorubicin and paclitaxel in metastatic patients increased RR to 47% compared to 36% and 34% of doxorubicin or paclitaxel alone, respectively. Interestingly, there was not significant benefit when drugs were administered sequentially [252].

However, TNBC patients who do not achieve pCR in response to therapy, and have residual disease, have decreased overall survival compared with non-TNBC patients who had residual disease. Specifically, TNBC patients have decreased PFS and overall survival within the first 3 years of diagnosis [253]. Overall, drug combinations have greater efficacy over single-agent treatment, but can be associated with increased toxicity, compared to single agent therapy [193, 194]. Therefore, although TNBC patients initially respond well to chemotherapy, their poor prognosis and increased risk of recurrence forces a search for novel therapies.

Table 7. Chemotherapy options for TNBC patients			
Drug Family	**Mechanism of action**	**Drugs**	**Response as single agent in breast cancer patients**
Anthracyclines	Intercalates with DNA, inhibits topoisomerase II activity	Doxorubicin	10-50%
		Epirubicin	13-48%
		Etoposide	15-35%
Anti-metabolites	Nucleoside analogs, disrupt RNA and DNA synthesis	5-fluorouracil	25-54%
		Capecitabine	20-35%
		Gemcitabine	12-37%
Taxanes	Microtubule stabilizer by binding to β-tubulin	Doxetaxel	21 days: 18-68% 7 days: 33-50%
		Paclitaxel	21 days: 16-62% 7 days: 22-53%
		Abraxane	33-48%
Vinca-alkaloids	Inhibit microtubule formation	Vinorelbine	25-50%
Platinum-based	Covalent binding to DNA purine bases	Cisplatin, carboplatin	9-51%
Adapted from: [193], [254-261]			

Several novel targeted therapies against TNBC are in clinical trials. PARP inhibitors are proving to be very promising against *BRCA1/BRCA2* mutation derived tumors. PARP repairs SSBs via BER, with PARP inhibition leading to a collapse of replication forks. This causes SSBs to develop into DSBs that would require HR for repair. However, studies found that cancer cells with *BRCA1/BRCA2* genes are deficient in HR, sensitizing cells to PARP inhibition [170, 262]. Since most BRCA-derived tumors are diagnosed as TN and TN tumors have many of the same DNA-repair deficiencies as *BRCA* tumors, PARP inhibitors became an obvious treatment strategy for TNBC. PARP inhibitors are also being evaluated for serous ovarian cancer. There are currently six PARP inhibitors being examined in the clinic, including olaparib, veliparib, rucaparib, BMN-673, CEP-9722 and niraparib [263]. As a single agent, PARP inhibitors have anti-tumor activity in *BRCA*-mutation carriers and non-carriers (Table 8). A phase II study in *BRCA*-mutation carriers with refractory disease found that olaparib monotherapy had a RR of 31.1% and 12.9% in ovarian and breast cancer patients, respectively [264]. Several trials have combined PARP inhibitors with chemotherapeutics, aiming to maximize DNA damage and tumor cell death (Table 8). Ongoing phase II/III trials, including combining rucaparib with cisplatin in TNBC patients with *BRCA*-mutations, will further illuminate the utility of PARP inhibitors in the clinic [263].

Table 8. Sample of clinical trials with PARP inhibitors				
Clinical trials independent on *BRCA* status				
NCT Identifier	**Regimens**	**Setting**	**Major Findings**	**Ref.**
NCT007535451	Olaparib: single agent	Randomized, double blind placebo-controlled phase II study for maintenance of recurrent platinum sensitive serous ovarian cancer	Well tolerated; PFS improved of 8.4 months vs. 4.8 months	[265]
NCT00679783	Olaparib	Phase II study in TNBC and serous ovarian cancer	OR in 41% of BRCA patients vs. 24% in non-*BRCA* patients (ovarian); no OR in BC patients	[266]
NCT00707707	Olaparib with Paclitaxel	Phase I study in metastatic TNBC patients	Diarrhea, nausea and neutropenia. 37% partial response	[267]
Clinical trials dependent on *BRCA* status				
NCT00628251	Liposomal doxorubicin and olaparib	Phase II study BRCA-patients with relapsed ovarian cancer	No statistical difference in treatment groups	[268]
NCT0144518	Carboplatin and olaparib	PhaseI/IIb study in BRCA-patients with breast and ovarian cancer	Well tolerated; 50% partial response, 1 patient pCR	[269]
NCT00749502	Niraparib	Phase I study (24/100 with *BRCA* mutations)	Well tolerated; 40% OR in *BRCA*-patients with ovarian cancer, 50% OR in *BRCA*-patients with BC	[270]

EGFR has become an attractive target against TNBC. Some studies have found up to 64% of TNBC tumors having overexpression of EGFR protein; with 33% of tumors having high *EGFR* gene copy number, correlating to poor outcome [271]. *In vitro* studies demonstrated increased sensitivity of BLBC cells to the monoclonal antibody cetuximab compared to luminal cells, with combination therapy of carboplatin followed by cetuximab being synergistic [272]. A randomized phase II study examining cetuximab alone versus cetuximab with carboplatin in TN MBC patients found that while cetuximab was well tolerated, it showed low activity against the tumor. However, combination therapy had an 18% RR, with 27% of patients showing clinical benefit [273]. However, more clinical trials are needed to validate the efficacy of targeting EGFR as both single-agent and combination therapy.

The vascular endothelial growth factor (VEGF) family and their receptors (VEGFR) are required for angiogenesis and have been linked to tumor growth and metastasis in variety of tumors, including breast cancer [193, 274]. As single-agent therapy, bevacizumab (Avastin), a humanized recombinant antibody against VEGF-A, had a RR of 6.7% in a phase I/II trial in MBC patients, with 22% of patients experiencing hypertension [275]. However a phase III trial in metastatic breast cancer patients found that paclitaxel plus bevacizumab versus paclitaxel alone increased PFS to 11.8 vs. 5.9 months and RR to 36.9% vs. 21.2%, respectively [276]. Moreover, a randomized study in TNBC patients with primary tumors found treatment with epirubicin and cyclophosphamide followed by docetaxel with and without the addition of bevacizumab increased pCR to 39.3% versus 27.9%, respectively [277]. Therefore, inhibition of angiogenesis can increase the response to chemotherapy in TNBC patients.

1.8 GAP IN KNOWLEDGE

The molecular and clinical characteristics of TNBC demonstrate that TNBC patients cannot benefit from currently approved targeted therapy. Initial sensitivity to

chemotherapeutics has not been able to greatly improve the overall survival and prognosis of TNBC patients. While novel treatments are in development, current studies do not investigate how targeting the deregulated TNBC cell-cycle checkpoints could be synthetically lethal with chemotherapeutics. In order to generate a novel treatment strategy the following questions must be answered:

- Would the deregulation of the cell cycle sensitize TNBC cells to cell cycle inhibitors; would non-TNBC and non-transformed cells respond differently?
- Can a synthetic lethal combination of cell-cycle inhibition and chemotherapeutics specifically target TNBC cells, without inflicting harm to non-transformed cells?
- Will combination cell cycle targeted therapy be an effective method in a pre-clinical model?
- What affect would combination therapy have on the cell cycle of TNBC cells compared to non-transformed cells?
- Is there a molecular target/pathway that can be used as a marker to predict combination treatment response?
- Can combination treatment augment the DNA damage inflicted by chemotherapeutics explicitly in TNBC cells?

The following chapters of this dissertation will address these questions. The overall hypothesis of this dissertation is that **TNBC cells are sensitive to cell cycle-targeted combination therapy, leaving non-transformed cells unharmed**. The examination of these questions, potentially leading to the generation of a novel targeted therapy treatment strategy, could greatly improve the care of TNBC patients.

CHAPTER 2: SEQUENTIAL ADMINSTRATION OF ROSCOVITINE FOLLOWED BY DOXORUBICIN INDUCES SYNERGISTIC CELL DEATH IN TNBC CELLS

2.1A DEREGULATION OF THE CELL CYCLE IN BREAST CANCER

As with all tumor cells, breast cancer cells possess the ability to proliferate continuously due to gene amplifications of cycling promoting factors, mutations in CKIs and alterations in protein expression. About 15% of breast cancer tumors have gene amplification at chromosome 11q13, where *CCND1* is located [278]. Moreover, 50% of breast cancers have high cyclin D1 expression at both the mRNA and protein level, contributing to Rb inhibition [279, 280]. Also, there is a positive correlation between ER positivity and cyclin D1 expression, with ER activity inducing cyclin D1 transcription [281, 282]. Unlike cyclin D1, cyclin E is seldom amplified in breast cancer. However, over 40% of breast tumors have cyclin E overexpression at the protein level, contributing to CDK2 overactivation [80, 283]. Cyclin E protein overexpression is a poor prognostic marker in breast cancer, and is associated with negative ER and PR status [80, 284]. As previously described, cytoplasmic localization of LMW-E is oncogenic in breast cancer, and has been shown to specifically enable ER positive breast cancer cells to bypass letrozole-induced G1 arrest [76, 285]. High expression of cyclin B1 in either the cytoplasm or the nucleus correlated with poor overall survival in breast cancer, suggesting that any increase in cyclin B1 expression can increase the aggressiveness of the tumor [286]. In addition to aberrant cyclin expression, CKIs can also be deregulated in cancer. The CKI p27 has decreased expression and irregular cytoplasmic localization in breast cancer cells. Moreover, decreased nuclear p27 expression correlated with high tumor grade, negative ER status and could serve as predictor of decreased disease free survival in breast cancer [287, 288]. About 20-30% of breast cancers have promoter hypermethylation, leading to epigenetic silencing of the CKI $p16^{INK4A}$ gene [289]. Whereas p21 is rarely mutated in breast cancer, the p21 transcription factor p53 is mutated in 54-82% of BLBC and TNBC [180, 290, 291].

Activity of kinases that participate in chromosome segregation can also be augmented in breast cancer. Aurora A kinase, required for chromosome segregation, is often overexpressed in breast cancer due to gene amplification, leading to chromosomal instability [292]. Importantly, microarray analysis on breast cancer tumors from patients under the age of 55 revealed that expression of several cell cycle genes, such as cyclin E2, cyclin B2, CDC25B, and Bub1, comprise a poor prognosis gene signature that predicts metastasis in less than 5 years [234]. Therefore, targeting the cell cycle is a viable treatment option for breast cancer, especially to target more aggressive tumors.

2.1B CYCLIN AND CDK KNOCKOUT MICE

Cyclin and CDK knockout mice were generated to investigate the role of each protein during development and to examine compensatory mechanisms within the cell cycle (Table 9). For example, cyclin A1 knockout mice are viable, with only male mice suffering from sterility. This suggests that cyclin A2 can compensate for cyclin A1, and that cyclin A1 is only required for spermatogenesis [293]. However, cyclin A2 knockout mice are embryonic lethal [294]. Similarly, while cyclin B1 knockout mice are embryonic lethal, cyclin B2 are viable, indicating that cyclin B1 can compensate for cyclin B2 during development. Knockout of other cyclins show abnormalities in specific tissues, indicating their participation in tissue-specific development (Table 9). Cyclin A and B are required to activate CDK1, with CDK1 knockout mice also being embryonic lethal [295]. Moreover it was found CDK1 could bind to cyclin E in CDK2-deficient MEFs and, in the absence of all interphase CDKS, can phosphorylate Rb to complete the cell cycle [295, 296]. Together, these studies indicate that CDK1 activity can drive the entire mammalian cell cycle in the absence of other CDKs [295]. However, the compensatory mechanisms observed in *in vivo* model systems do not always translate to CDK inhibition studies *in vitro*. This may be because CDK inhibition does not automatically make their cyclin subunits accessible to bind to other CDKs.

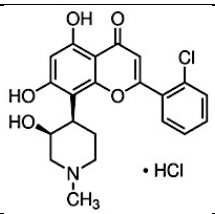
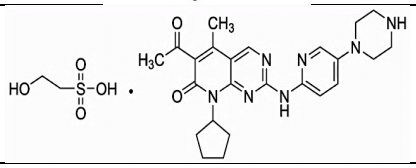
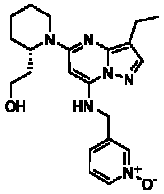
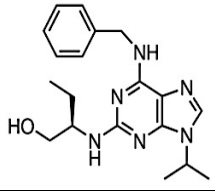
Therefore, *in vitro* systems are often preferred for development of CDK inhibitors as opposed to transgenic models [33].

Table 9. Phenotypes of cyclin and CDK knockout mice		
Cyclin/CDK	**Phenotype**	**Ref.**
Cyclin A1	Viable, no abnormalities. Only male mice sterile	[293]
Cyclin A2	Embryonic lethal at E5.5	[294]
Cyclin B1	Embryonic lethal at E10.5	[297]
Cyclin B2	Viable, no abnormalities, both genders fertile.	[297]
Cyclin D1	Viable, reduced body size, neurological abnormalities, hypoplastic retina, mammary gland defects (inability to lactate during pregnancy)	[298, 299]
Cyclin D2	Viable, females sterile and males with hypoplastic testes. Defects in B-lymphocyte proliferation, pancreatic β-cell proliferation, hypoplastic thymus and abnormalities in cerebellar development and adult neurogenesis	[300-302]
Cyclin D3	Viable. Defects in T-lymphocyte development	[303]
Cyclin D1/D2	Reduced body size, hypoplastic cerebella, mortality 3 weeks after birth	[304]
Cyclin D2/D3	Megaloblastic anemia, embryonic lethal at E17.5	[304]
Cyclin D1/D2/D3	Proliferative defects in hematopoietic cells and cardiac myocytes, embryonic lethal at E16.5	[305]
Cyclin E1	Viable, no abnormalities	[306]
Cyclin E2	Viable, no abnormalities, reduced male fertility	[307]
Cyclin E1/E2	Defects in extraembryonic tissues, embryonic lethal at E11.5	[307]
CDK1	Embryonic lethal E2.5	[295]
CDK2	Reduced body size, impaired neural progenitor cell proliferation, both genders sterile	[308, 309]
CDK4	Reduced body size, insulin deficient, diabetes caused by reduced pancreatic β-cells,	[310]
CDK6	Hypoplasia and defects of thymus and spleen	[311]
CDK2/4	Heart defects, embryonic lethal at E15.5	[312]
CDK2/6	Reduced body size, hematopoietic defects, both genders sterile	[311]
CDK4/6	Severe anemia, embryonic lethal at E14.5-E18.5	[311]
CDK2/4/6	Heart defects, hematopoietic defects, embryonic lethal at E13.5	[295]
CDK5	Severe neurological defects, mortality immediately following birth	[313]
CDK11	Embryonic lethal at E3.5	[314]
Adapted from: [33]		

2.1C CDK INHIBITORS IN CANCER

CDK inhibitors have been developed to target the consistent deregulation of the cell cycle in cancer. The two main types of CDK inhibitors are ATP-competitive inhibitors and non-ATP inhibitors. ATP-competitive inhibitors bind to the CDK catalytic ATP-site, preventing CDK activation. ATP-competitive inhibitors consist of heterocyclic families, including natural products (i.e. flavones and staurosporine), purines, pyrimidines, indoles, pyrazoles and thiaozoles (Table 10) [315]. The following section will detail ATP-competitive CDK inhibitors that have been extensively examined clinically (Table 11).

Table 10. Clinically examined CDK inhibitors

Drug (Company)	Drug Family	Structure	Target CDKs
Flavopiridol (Sanofi-Aventis)	Flavone		CDK1, CDK2, CDK4, CDK6, CDK7 & CDK9
Palbociclib (Pfizer)	Pyridopyrimidine		CDK4/CDK6
Dinaciclib (Merck)	Pyrazolo-pyrimidine		CDK1, CDK2, CDK5 & CDK9
Roscovitine (Cyclacel)	Purine		CDK1, CDK2, CDK5, CDK7, & CDK9

Adapted from: [315] Chemical structures adapted from Sigma-Aldrich and Selleckchem

Table 11. Clinical trials with CDK inhibitors			
Drug	**Trial Phase**	**Results**	**Ref.**
Flavopiridol	I	Solid tumors: complete response/partial response in 5/27 patients, stable disease in 10/27 patients (C)	[316]
	I	CLL: partial response in 40% of patients, PFS of 12 months (M)	[317]
	II	Malignant melanoma: no clinical significance (M)	[318]
	II	NSCLC: No OR, disease progression in 4/20 patients (M)	[319]
	II	AML: With cytrabine and mitoxantrane increased complete remission compared to standard of care (C)	[320]
Palbociclib	I	Advanced Rb-positive solid tumors: In a 28-day cycle (3 weeks on, 1 week off), 125 mg/daily is MTD. SD in 35% of patients (M)	[321]
	II	Advanced Rb-positive BC: 38% of patients had SD for over 6 months, with 19% of patients having some clinical benefit. Over 50% of patients had dose reduced because of cytopenia (M)	[322]
	II	Advanced ER-positive BC: Improved PFS from 10.2 vs. 20.2 months comparing letrozole vs. letrozole plus palbociclib, respectively (C vs. M)	[323]
	III	ER positive BC: on-going with letrozole (C vs. M)	NCT01740427
Dinaciclib	I	10/48 patients, including (NSCLC, prostate cancer sarcoma, melanoma, esophageal carcinoma, gastrointestinal stromal tumor, adenoid cyst carcinoma and pseudomxyoma peritonei) achieved SD for at least four 28-day treatment cycles (M)	[324]
	II	Advanced breast cancer: 2/7 patients had PR, no benefit compared to capecitabine (TTP 2.73 vs. 4.17 months) (M)	[325]
	II	NSCLC: TTP 1.49 vs. 1.58 months, dinaciclib vs. erlotinib, respectively. Adverse effects included neutropenia, leukopenia, vomiting and diarrhea (M)	[326]
	III	CLL: dinaciclib vs. Ofatumumab, on-going (M)	NCT01580228
Roscovitine	I	Nasopharyngeal cancer: 50% of patients had tumor reduction (M)	[327]
	I	NSCLC: In combination with gemcitabine and cisplatin. Patients showed PR (6/27), SD (7/27) and disease progression (1/27). MTD 800 mg (C)	[328]
	I	Advanced solid tumors: Sequential combination treatment sapacitabine and roscovitine induced PR 2 patients with breast and pancreatic cancer and SD in 6 patients. Germline BRCA mutation suggested as marker to response (C)	[329]
M = monotherapy, C = combination therapy			

Flavopiridol is a semisynthetic flavone analog of an Indian plant rohutkine. Flavopiridol has shown to have activity against CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9 at nanomolar concentrations and has also been shown to decrease cyclin D1 mRNA expression. Flavopiridol is administered intravenously [290, 330]. In vitro studies performed on multiple myeloma cell lines found that flavopiridol induces apoptosis while reducing mRNA and protein levels of the anit-apoptotic protein MCL-1 [331]. However, this CDK inhibitor has shown mixed results in the clinic (Table 11). Untreated metastatic melanoma patients enrolled in a phase II study showed no significant clinical benefit with single-agent flavopiridol. Although flavopiridol was relatively well tolerated, over 80% patients suffered from diarrhea [318]. Similarly, a phase II trial in NSCLC patients showed no objective response, with some patients progressing during treatment [319]. Phase II studies in patients with acute myeloid leukemia who were treated with flavopiridol followed by cytarabine and mitoxantrone had increased complete remission rates compared to patients treated with cytarabine and danurubicin [320]. Moreover, a phase I trial in patients with solid tumors found that combination of docetaxel followed by flavopiridol induced complete response or partial response in 5/27 patients and stable disease (SD) in 10/27 patients [316]. These studies illustrate the clinical potential of flavopiridol in combination therapy. Although flavopiridol has shown promising results in leukemias, its manufacturer, Sanofi-Aventis, is no longer developing it for cancer treatment [315].

Palbociclib is a potent CDK4/CDK6 inhibitor that has promising therapeutic potential in breast cancer [332]. While other CDK4/CDK6 inhibitors, such as LEE011 and LY283519 have been clinically examined, palbociclib is the focus here due to the success and efficacy it has made in breast cancer (Table 11) [333]. An *in vitro* study that examined the response of forty-four human breast cancer cell lines and three immortalized cell lines to palbociclib treatment found that luminal ER positive (including HER2 amplified) cells were the most sensitive to treatment compared to BLBC or cells that had undergone epithelial to

mesenchymal transition. Growth inhibition was measured via cell counting, as no cytotoxicity was detected. Moreover, sensitive cells had elevated gene expression of Rb and cyclin D1 and decreased levels of p16. Palbociclib treatment reduced phospho-Rb specifically in sensitive cell lines, causing a G0/G1 arrest. Additionally, palbociclib increased the growth inhibitory affect of tamoxifen and trastuzumab treatment in ER-positive and HER2 amplified cell lines, respectively [334]. When examined in drug resistant cell lines, palbociclib treatment re-sensitized cells to tamoxifen and induced growth arrest and senescence in cells resistant to endocrine therapy [334, 335]. A phase I study that enrolled patients with Rb-positive advanced solid tumors refractory to standard treatment examined treatment with palbociclib in a 3 weeks on, 1 week off schedule, found that 125 mg once daily was the maximum tolerated dose (MTD) and induced SD in 13/41 [321]. A phase II trial enrolled 37 women with advanced breast cancer with confirmed Rb-positive protein expression also examined the dose of 125 mg/daily 3 weeks on, 1 week off schedule. Fifty-one percent had dose reductions due to cytopenia. Thirty eight percent of patients had SD for over 6 months, with 19% of patients having some clinical benefit. However, Rb nuclear expression, percent Ki-67, cyclin D gene amplification and p16 loss did not correlate to a positive response, indicating that further investigation is required for predictive response marker [322]. A randomized phase II study investigated the safety and efficacy of letrozole versus letrozole plus palbociclib in treatment-naive postmenopausal women with advanced ER positive (HER2 negative) breast cancer. Letrozole was administered continuously at 2.5 mg daily, while palbociclib was administered 125 mg daily for 3 weeks on, 1 week off. This study found that combination treatment significantly improved the median PFS from 10.2 versus 20.2 months comparing the letrozole group versus the letrozole plus palbociclib, respectively. For toxicity, 1% versus 54% of patients had neutropenia, and none versus 19% had leucopenia with letrozole alone versus letrozole plus palbociclib, respectively. Similar to other studies, this trial did not find any correlation between cyclin D1 and p16 status and

response [323]. Based on the significant phase II results, phase III trials on palbociclib in combination with letrozole are currently ongoing in ER-positive breast cancer [336].

Dinaciclib, or SCH727965, is a potent pyrazol-pyrimidine derivative that inhibits CDK1, CDK2, CDK5 and CDK9 in the nanomolar range [330]. Dinaciclib has shown activity against a broad range of malignancies in pre-clinical settings. *In vitro* studies with ovarian cancer cells found that dinaciclib was more effective at reducing phospho-Rb (S807/811) expression while inducing apoptosis measured by cleaved PARP1 expression at >6.25 nM compared to 1μM flavopiridol [337]. A human ovarian cancer xenograft in a mouse model system found that dinaciclib had a higher MTD of 60 mg/kg versus <10 mg/kg of flavopiridol (based on 20% weight loss) [337]. Furthermore, dinaciclib induced a 50% reduction in tumor growth at 5 mg/kg after 7 days of treatment, versus 10 mg/kg of flavopiridol. Additionally, dinaciclib decreased pancreatic cancer cell viability and migration capacity *in vitro*. Human pancreatic cancer xenograft studies revealed that dinaciclib reduced tumor growth both as a single agent and in combination with gemcitabine [338]. Since flavopiridol, which showed promise in leukemia, was discontinued, dinaciclib was examined in a preclinical model against CLL cells. A 2hr exposure of a clinically achievable dose of dinaciclib induced apoptosis in CLL cells isolated from patients, including high-risk patients [339]. Dinaciclib has also been investigated in clinical trials (Table 11). A phase-I dose escalation study in patients with solid tumors found the maximum administered dose to be 14 mg/m^2, recommending a phase II dose of dinaciclib to be 12 mg/m^2. In this study, 10/48 patients, including patients with NSCLC, prostate cancer, sarcoma and melanoma, achieved disease stabilization for at least four 28-day treatment cycles [324]. A phase II open-label study comparing standard of care treatment erlotinib to dinaciclib in NSCLC patients found that time to progression (TTP) was 1.58 months for erlotinib versus 1.49 months for dinaciclib. These patients experienced neutropenia, leukopenia and vomiting [326]. Furthermore, a phase II study in patients with advanced breast cancer found that dinaciclib

treatment of 50 mg/m^2 once every 21 days caused a partial response in 2/7 patients. However, dinaciclib treatment had earlier TTP compared to capecitabine treatment (2.73 vs. 4.17 months), and the study concluded after 30 days ([325]. While showing limited efficacy in solid tumors, the safety and efficacy of dinaciclib compared to ofatumumab (a monoclonal antibody against CD20) is being investigated in a phase III trial in refractory CLL patients (NCT01580228) [336]. Similar to other CDK inhibitors, the narrow efficacy of dinaciclib as single-agent therapy against solid tumors could indicate that it could be more efficacious in combination therapy.

Roscovitine, also known as selicicib or CY-202, is also a pan-CDK inhibitor that has activity against CDK1, CDK2, CDK5, CDK7 and CDK9 at micromolar concentrations according to *in vitro* kinase assays [330]. Roscovitine has been used in combination in multiple pre-clinical models. In p53 mutant breast cancer cells roscovitine increased accumulation in G2 and augmented ionizing radiation (IR)-induced growth inhibition. Moreover, concomitant treatment of roscovitine (100mg/kg) and IR (7.5Gy) significantly reduced tumor volume compared to either treatment alone in a xenograft model system. Here, it was suggested that combination treatment reduced the ability of cells to undergo NHEJ [340]. Combination treatment of roscovitine and IR also significantly increased apoptosis of NSCLC cells *in vitro*. Moreover, combination treatment reduced expression of NHEJ proteins Ku70 and Ku80, again suggesting that roscovitine can inhibit DNA repair [341]. Roscovitine also synergized with IR in nasopharyngeal cancer cells by increasing apoptosis and inducing a G2/M cell cycle arrest, while retarding tumor growth in a xenograft model [342]. In addition to IR, roscovitine has been examined in combination with chemotherapeutics. Our lab has found that roscovitine synergizes with doxorubicin to induce cytotoxicity in sarcoma cell lines *in vitro* [343].

Roscovitine has been investigated clinically where it was administered orally (Table 11) [315]. A phase I study that examined treatment with roscovitine, gemcitabine and

cisplatin in NSCLC patients found that the maximum tolerated dose of roscovitine was 800 mg twice daily (with 1000 mg/m2 gemcitabine and 75mg/m2 cisplatin). In this study, 6/27 patients had a partial response, 7/27 had SD and 1/27 patients had disease progression [328]. In a phase I study of nasopharyngeal cancer, roscovitine was administered at either 400 mg or 800 mg twice daily on days 1 to 3 and 8 to 12. While patients receiving 400 mg did not have significant toxicities, patients receiving 800 mg had liver toxicity and vomiting. However, despite being only briefly exposed, 50% of evaluable patients showed tumor reduction; with IHC analysis revealed increased apoptosis and necrosis and decreased cyclin D1, MCL1 and phospho-Rb [327]. Moreover, a phase I study examined the toxicity and efficacy of sequential administration of sapacitabine and roscovitine in patients with advanced solid tumors [329]. Sapacitabine is a prodrug of the nucleoside analog 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC), that can induce SSBs that develop into DSBs [344]. These patients received sapacitabine daily for days 1-7, roscovitine days 8-10, followed by 11 days off. Here the MTD for roscovitine (in combination with sapacitabine) was found to be 1200 mg, with dose limiting toxicities being reversible neutropenia and increased transaminase levels. Of the 27 patients, two patients with either breast or pancreatic cancer, both BRCA mutation carriers, had a partial response. Additionally, 6 patients had SD for at least 12 weeks, including a BRCA mutation carrier patient with ovarian cancer for over 24 weeks. It was suggested that BRCA mutations could be a marker for response to the sapacitabine and roscovitine combination [329]. Overall, more studies are needed to confirm the clinical efficacy of roscovitine, especially in combination with DNA damaging agents.

2.1D HYPOTHESIS AND SPECIFIC AIMS

Despite showing encouraging results when combined with DNA-damaging agents (and targeted therapies), little has been done to develop a combination treatment strategy in TNBC with CDK inhibitors. While others have shown that over-activation of the oncogene

Myc causes sensitivity to CDK inhibition in TNBC cells, this strategy again only examines CDK inhibition in a monotherapy setting [345]. As previously described, due to defects in cell cycle regulation, cancer cells continuously proliferate even in the presence of DNA damage, with TNBC tumors initially responding to chemotherapeutics (Table 2). Anthracyclines, including topoisermase II inhibitors doxorubicin and epirubicin, are used in the standard of care of TNBC and are often used in combination therapy (Table 7) [179]. Therefore, we *hypothesized that combining CDK inhibitor roscovitine with the chemotherapeutic doxorubicin would cause increased cell death only in TNBC cells*. To address this hypothesis the following, specific aims were examined:

- Determine the cell cycle response of HMEC, ER positive and TNBC cells to roscovitine treatment.
- Investigate the inhibitory effect of combination treatment of roscovitine and doxorubicin on HMEC and TNBC cells.
- Examine combination-induced cytotoxicity in TNBC and HMEC cells.
- Establish combination treatment in a pre-clinical *in vivo* model system.

Overall, the data presented in this chapter demonstrates that roscovitine treatment induced a significant G2/M arrest specifically in TNBC cells. Analysis using the Chou-Talalay method revealed that only sequential administration of roscovitine followed by doxorubicin induced synergistic cell inhibition only in TNBC cells, not in HMEC cells [346]. Moreover, administration of roscovitine prior to doxorubicin increased apoptosis only in TNBC cells compared to single drug treatment. Finally, a xenograft model of TNBC cells found that combination treatment was well tolerated and significantly decreased tumor growth and increased overall survival compared to single agent treatment.

2.2 MATERIALS AND METHODS

2.2A CELL CULTURE

The immortalized human mammary epithelial (HMEC) cell lines 76NE6 and 76NF2V were obtained from Dr. V. Band (University of Nebraska Medical Center) and MCF10A cells were obtained from the American Type Cell Culture Collection (ATCC; Manassas, VA). These cells were maintained in DCFI-1 medium in culture as described previously [347]. The breast cancer cell lines MDA MB 157, MDA-MB-231 and HCC1806 cells were obtained from the ATCC and maintained in complete alpha medium [347]. The breast cancer cell lines MCF-7, ZR75-1, T47D and MDA MB 468 was obtained from the ATCC and maintained in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. All cells were kept in a humidified incubator in a 6.5% CO_2 atmosphere at 37℃. All cells were tested for mycoplasmal contamination, and their identities were verified via karyotype analysis. Breast cancer cell line hormone receptor, HER2 and p53 and Rb status are available in Table 12.

Table 12. Breast cancer cell lines examined

Cell Line	ER	PR	HER2	p53 status	Rb status
MCF10A	-	-	-	WT	WT
76NE6	-		-	Degraded by HPV-6	WT
76NF2V	-	-	-	WT	WT
HCC1806	-	-	-	Mutant	Functionally inactive
MDA MB 157	-	-	-	Mutant	Functionally inactive
MDA MB 231	-	-	-	Mutant	Functionally inactive
MDA MB 468	-	-	-	Mutant	-
MCF7	+	+	-	WT	WT
T47D	+	+	-	Heterozygous Mutant	WT
ZR75-1	+	-	+	WT	WT

Adapted from: [347, 348]

2.2B DRUGS

Roscovitine, provided by Dr. Laurent Meijer (National Center for Scientific Research, Paris France), was diluted to 10 mM in dimethyl sulfoxide (DMSO). Doxorubicin-HCl (Bedford Laboratories, Bedford, OH) was reconstituted in a 0.9% sterile saline solution at 2 mg/mL and shielded from light. Staurosporine (Sigma-Aldrich) was diluted to 2.14 mM in DMSO.

2.2C FLOW CYTOMETRY

Cells were seeded in 10 cm dishes at 3.5 x 10^5 cells per plate to examine cell-cycle phases in response to drug treatment. Cells were then treated with roscovitine at 20 μM for 24 hours. Following treatment, cells were fixed and stained to measure their DNA content as described previously [285]. Briefly, cells were resuspended and fixed in 1.5 mL of cold phosphate-buffered saline (PBS) and 3.5 mL of cold ethanol overnight. Cells were then washed with PBS, resuspended in PBS containing 10 μg/mL propidium iodide, RNase A, Tween 20, and bovine serum albumin and incubated at 4°C overnight. Prior to measuring their DNA content, cell samples were incubated for 1 hour at 37°C. Samples were analyzed at The University of Texas MD Anderson Cancer Center Flow Cytometry and Cellular Imaging Facility using a Beckman Coulter Gallios Flow Cytometer (Indianapolis, IN) equipped with the Kaluza software program (Beckman Coulter).

2.2D HIGH-THROUGHPUT SURVIVAL ASSAY

To assess the effect of combination drug treatment on HMEC and TNBC cell lines, cells were subjected to a high-throughput survival assay (HTSA) as described previously (Figure 3) [349]. Briefly, for all combinations cells were seeded in 96-well plates (Table 13). We administered the roscovitine and doxorubicin combination drug treatment simultaneously and sequentially, in both directions (Figure 3 A, B). For concomitant drug administration, cells were treated for 72 hours with roscovitine and doxorubicin

simultaneously (Table 14, R+D). For sequential drug administration, cells were treated with drug A at the 10% inhibitory concentration (IC_{10}), IC_{25}, and IC_{50} for 24 hours. Following drug A treatment, the medium was removed from the wells, and cells were treated with drug B at the IC_{10} to the IC_{50} for 48 hours, equaling 72 hours of total drug treatment (Table 14, D→ R, R→D). Following the completion of 72 hours of drug treatment, the medium in the wells was replaced with fresh-drug free medium. Every 96-well plate contained cells that remained untreated as controls. Controls for treatment with drug A or drug B only were also included. The drug-free medium was changed every 48 hours for 9 days. Nine days after the removal of drug B, the plates were subjected to a (3-(4, 5)-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (2.5 mg/mL; Sigma-Aldrich), incubated for 4 hours at 37℃, and solubilized (0.04 N HCl and 1% sodium dodecyl sulfate [SDS], in isopropyl alcohol). Absorbance was read at 590 nM using Epoch microplate spectrophotometer with the Gen5 software program (BioTek, Winooski, VT). Isobolograms and combinational indices were generated using the CalcuSyn software program (Biosoft, Cambridge, UK).

Table 13. Cell number/well seeded for HTSA

Cell Line	Cells/well
MCF10A	1000
MDA MB 157	3500
MDA MB 231	1500
MDA MB 468	2000

Table 14. Drug concentrations used for combination treatment

	R+D		D→R		R→D	
Cell Line	**R (μM) $IC_{10, 25, 50}$**	**D (nM) IC_{10}-IC_{50}**	**D (nM) $IC_{10, 25, 50}$**	**R (μM) IC_{10}-IC_{50}**	**R (μM) $IC_{10, 25, 50}$**	**D (nM) IC_{10}-IC_{50}**
MCF10A	7,10,18	6-13	12,22,30	1-12	9, 11, 13	1-20
MDA MB157	5,9,15	4-8	9.5,12,17.5	4-26	8, 22, 32	1-15
MDA MB 231	5,18, 25	5-18	1,3,6	2-20	6, 18, 24	5-42
MDA MB 468	10,12,15	5-9	1, 22, 25	1-16	1, 10, 24	2-21

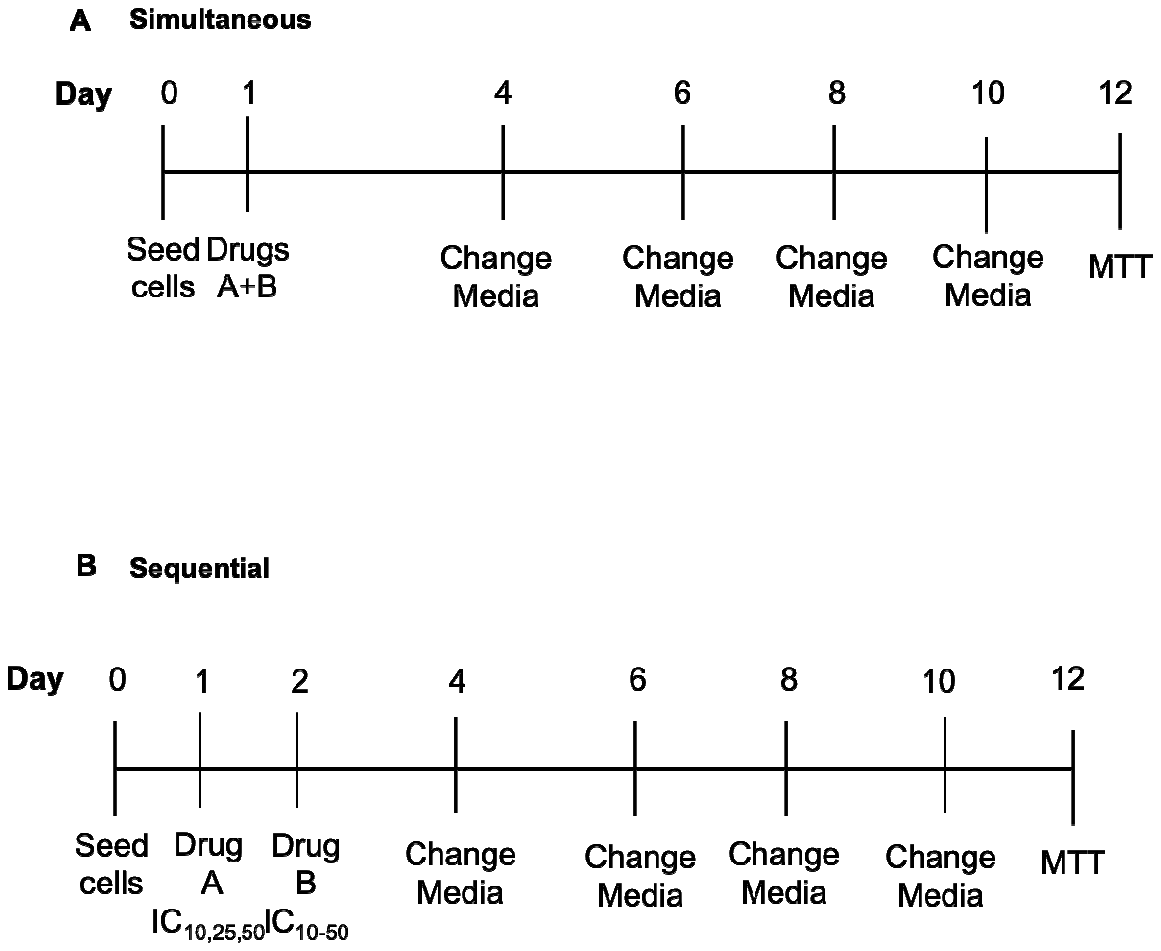


Figure 3. Schematic illustrating the HTSA using both simultaneous (A) and sequential (B) drug administration

2.2E DYE-EXCLUSION ASSAY

To examine cell viability, cells were subjected to a dye-exclusion assay. Cells were seeded at 3.5 x 10^5 cells per plate and treated with roscovitine for 24 hours, followed by doxorubicin for 48 hours at IC_{50} concentrations (Table 14). Roscovitine and doxorubicin only treated cells were used as single drug controls. Untreated and staurosporine-treated cells served as negative and positive controls, respectively. Cells were harvested at indicated times, and cell pellets were washed with cold PBS. Following centrifugation, pellets were resuspended in PBS, and propidium iodide was added to each harvested sample at a final concentration of 2 μg/mL prior to flow cytometric analysis using an LSR II flow cytometer equipped with the CellQuest Pro software program (BD Bioscience).

2.2F CASPASE ACTIVITY LUCIFERASE ASSAY

A Caspase-Glo 3/7 (Promega, Madison, WI) assay was used to measure caspase activity in HMEC and TNBC cells in response to single and combination drug treatment. Cells were seeded in 96-well white-walled plates (Table 13). After 24 hours, cells were treated with roscovitine and doxorubicin at the IC_{50} concentrations for 24 and 48 hours, respectively (Table 14). Caspase 3/7 activity of the cells was measured after single (roscovitine or doxorubicin) and combination (roscovitine followed by doxorubicin) treatment according to the assay manufacturer's protocols. The luciferase activity was deteced using a Synergy H4 hybrid microplate reader equipped with the Gen5.1.1 software program (BioTek). Readings of wells containing media only were subtracted from all experimental values.

2.2G WESTERN BLOT ANALYSIS

To extract protein lysates from cells for Western blot analysis, cells were harvested following indicated treatments. Cells were washed with cold PBS, trypsinized (0.25%), and centrifuged. Next, cell pellets were washed with cold PBS and centrifuged again. Cell pellets were lysed with RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.3, 0.1% SDS, 1% Triton X-

100, 1% deoxycholate, and 5 mM ethylenediaminetetraacetic acid) containing protease inhibitors, with occasional vortexing. Cell pellets were centrifuged at 16,000 rpm for 60 minutes, with supernatant containing protein lysates. Western blot analysis was performed to examine protein expression in HMEC and TNBC cells as described previously [350]. Briefly, the protein lysate was subjected to electrophoresis on an SDS-polyacrylamide electrophoresis gel and transferred to Immobilon-P membranes overnight at 4℃ at 35 mV. Blots were blocked with BLOTTO milk for 1 hour at room temperature and incubated with primary antibody overnight at 4℃. Antibodies against PARP-1 (Cell Signaling Technology, Beverly, MA) and actin (EMD Millipore, Billerica, MA) were used to probe for protein expression. Blots were incubated with goat anti-rabbit or goat anti-mouse immunoglobulin-horseradish peroxidase-conjugates at a dilution of 1:5000 in BLOTTO for 1 hour (Pierce, Rockford, IL). Blots were then washed and developed using a Renaissance chemiluminescence system (Perkin Elmer Life Sciences, Inc., Boston, MA) as directed by the manufacturer. Image J was used to perform densitometry analysis.

2.2H TRANSFECTION

To generate transient knockdown cells, HMEC and TNBC cells were seeded for 96-well plates for survival analysis or 6-well plates for flow cytometry or Western blot analysis according to manufacture protocols (Thermo Scientific Transfection Dhramafect siRNA Transfection Protocol). Thermo Scientific siGENOME smart pool siRNA, including siControl pool #1, siCDK1 and siCDK2, were resuspended according to the manufacture's protocol at 100 µm. Cells were transfected with siRNA targeting CDK1, CDK1 or both using the transfection reagent Dharmafect formulation 1 according to the manufacture's protocol (Thermo Scientific). Non-coding siControl pool #1 was used as a negative control. Cells were harvested 48 hours post transfection or as indicated for Western blot analysis and flow cytometry. Cells were subjected to MTT for survival analysis 9 days after removal of doxorubicin.

2.2I XENOGRAFT MODEL

To develop a pre-clinical model, four-week old nude mice were injected with 4.5 million MDA MB 468 cells in a 1:2 ratio with matri gel (BD Bioscience) into mammary fat pads four and nine of the mouse. Once the tumor reached a volume (L x W^2/2) of 100-150 mm^3, mice were treated with vehicle + vehicle, roscovitine (50 mg/kg) 4 days on/3 days off, doxorubicin (2mg/kg) once a week, or 4 days of roscovitine followed by one day of doxorubicin for four cycles. All drugs were administered i.p. Roscovitine was diluted at 100 mg/ml in DMSO and then further diluted to 10 mg/ml in a carrier solution consisting of 10% Tween 80 (Sigma Aldrich), 20% N-N-dimethylacetamide (Acros Organics, Geel, Belgium) and 70% polyethylene glycol 400 (Sigma-Aldrich) [351]. Tumor volume and weight were measured twice a week. Mice were sacrificed if their total tumor burden reached 1500 mm^3, tumors became ulcerated or inhibited mouse movement or if mice loss 20% of initial body mass. Mice were housed five per cage in sterilized micro-isolator cages (Lab Products, Seaford, DE) furnished with corncob bedding. Mice received care in accordance with the Animal Welfare Act, the National Institutes of Health "Guide for the Care and Use of Laboratory Animals," and the institutional guidelines of the MD Anderson Cancer Center.

2.2J WESTERN BLOT ANALYSIS OF TUMOR TISSUE

Following surgical resection, tumors were snap frozen in liquid nitrogen. Protein lysates were extracted via sonication in a protease inhibitor solution as previously described [352]. Homogenates were centrifuged at 45,000 g for 45 minutes. Supernatants were subjected to Western blot analysis. Blots were probed with anti-PARP1 antibody (Cell Signaling Technology, Beverly, MA).

2.2K STATISTICAL ANALYSIS

The Student *t*-test with a 95% confidence interval was performed to determine p values. P values less than 0.05 were considered significant. Survival analysis was performed using the Mantel-Cox test.

2.3 RESULTS

2.3A ROSCOVITINE INDUCES A G2/M ARREST IN TNBC CELLS

To investigate how breast cancer subtype and G1 checkpoint status (Table 12) would affect response to CDK inhibition, the HMEC cell line MF10A, the ER positive cell line MCF7 and the TNBC cell line MDA MB 231 were treated with roscovitine for 24 hours followed by cell cycle analysis via flow cytometry. p53 wildtype HMEC and ER positive cells had 70 or 60% of cells in G1, respectively. However, over 60% of p53 mutant MDA MB 231 cells accumulated in the G2/M phase (Figure 4A). To examine the effect of CDK inhibition on G2/M accumulation, a wide panel of HMEC (MCF10A, 76NF2V and 76NE6), ER positive cells (MCF7, T47D and ZR75-1) and TNBC (MDA MB 157, MDA MB 231, MDA MB 468 and HCC1806) cells were treated with roscovitine for 24 hours followed by cell cycle analysis. Roscovitine induced a 10 percent or less increase in G2/M phase in HMEC cells MCF10A and 76NF2V. However, roscovitine caused a significant (p-value < 0.001) 20% increase in the p53 inactive 76NE6 cells (Figure 4A, B). Similar to HMEC cells, roscovitine induce a 10% or less increase in G2/M of ER positive cells. In comparison, all four TNBC cell lines exhibited a significant (p-value < 0.05) increase in the G2/M phase in response to treatment, with MDA MB 231 cells having over a 25% increase in cells in G2/M (Figure 4B, C). Roscovitine treatment enriched the G2/M population of the TNBC cell lines examined to 40-60 percent, compared to the non-TNBC cells with 30 percent or less accumulating in G2/M (Figure 4C). 76NE6 cells were the exception to the trend detected in HMEC cells, with 60% of cells accumulating in the G2/M phase following roscovitine treatment (Figure 4C). These data suggests that the roscovitine-induced G2/M arrest is subtype and p53 mutant status specific.

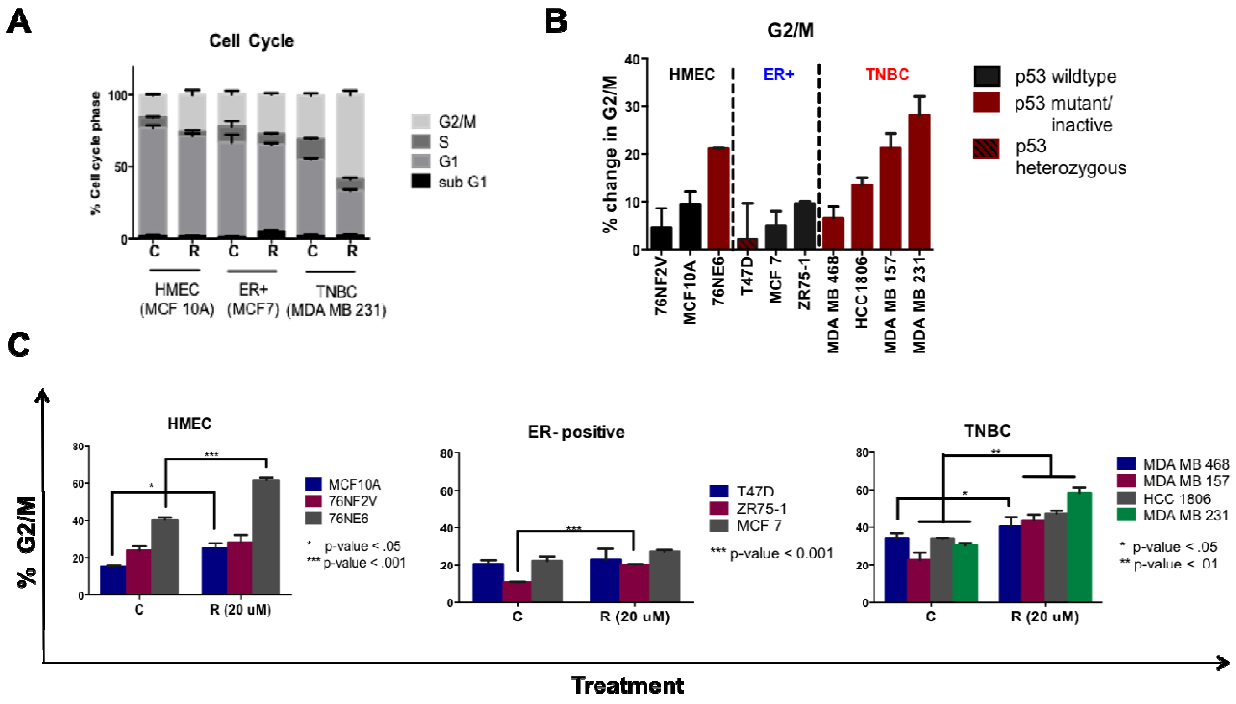


Figure 4. Roscovitine-induced G2/M accumulation is cell-type and p53 status specific. A, HMEC, ER-positive and TNBC cells were treated with roscovitine (R) for 24 hours followed by cell cycle analysis via flow cytometry. Untreated cells served as a control (C). B, Percent change in G2/M phase cells due to roscovitine treatment. C, Cell cycle analysis of a panel of HMEC, ER positive and TNBC cells in response to roscovitine. Percent of cells G2/M phase plotted. Mean ± SD.

2.3B SEQUENTIAL ADMINSTRATION OF ROSCOVITINE FOLLOWED BY DOXORUBICIN INDUCES SYNERGISTIC CELL INHIBITION ONLY IN TNBC CELLS

To investigate if the roscovitine-induced G2/M arrest in TNBC cells can be exploited to develop combination therapy, we subjected TNBC and HMEC cells to HTSA. We combined the clinically available chemotherapeutic doxorubicin with roscovitine both concomitantly and sequentially (Figure 3A, B). CalcuSyn, which quantifies synergy using the Cho-Talalay method, generates combination indices (CI) that determine if a combination is antagonistic (CI >1), additive (CI = 1) or synergistic (CI<1) [346]. The CI values demonstrated that concomitant treatment induced an antagonistic response in all cell lines (Figure 5A, left panel). Sequential administration of doxorubicin treatment prior to roscovitine also induced antagonism in all cell lines (Figure 5A, middle panel). Strikingly, administration of roscovitine prior to doxorubicin induced synergism in TNBC cell lines, but antagonism MCF10A cells (Figure 5A, right panel). Sequential administration of roscovitine and doxorubicin had a significantly lower (p-value < 0.05) CI value in all TNBC cell lines compared to simultaneous treatment or administration of doxorubicin prior to roscovitine (Figure 5A). Therefore, combination treatment can specifically inhibit TNBC cells and not HMEC cells.

Since roscovitine can inhibit multiple CDKs, we examined whether inhibition of CDK1, CDK2 or both CDKs simultaneously was required for synergism with doxorubicin treatment. MCF10A and MDA MB 468 cells were transiently transfected with siRNA against either CDK or both, with non-targeting siRNA used as a control. Cell viability was assessed on day 12 with MTT. While knockdown of CDK1 or both CDKs reduced cell viability in MCF10A cells by 60%, there was no further reduction in viability with the addition of doxorubicin. Knockdown of CDK2 did not decrease percent viability, however the addition of doxorubicin again reduced viability by 60% in MCF10A cells (Figure 5B). Transient knockdown of CDK1 or both CDKs simultaneously reduced viability by about 50% in MDA

MB 468 cells, whereas knockdown of CDK2 caused only 20% cell inhibition. However, the addition of doxorubicin to CDK1 or CDK1/CDK2 knockdown caused 90% cell inhibition in MDA MB 468 cells (Figure 5B). This suggests that inhibition of CDK1 combined with doxorubicin treatment is sufficient to induce synergistic cell inhibition in TNBC cells.

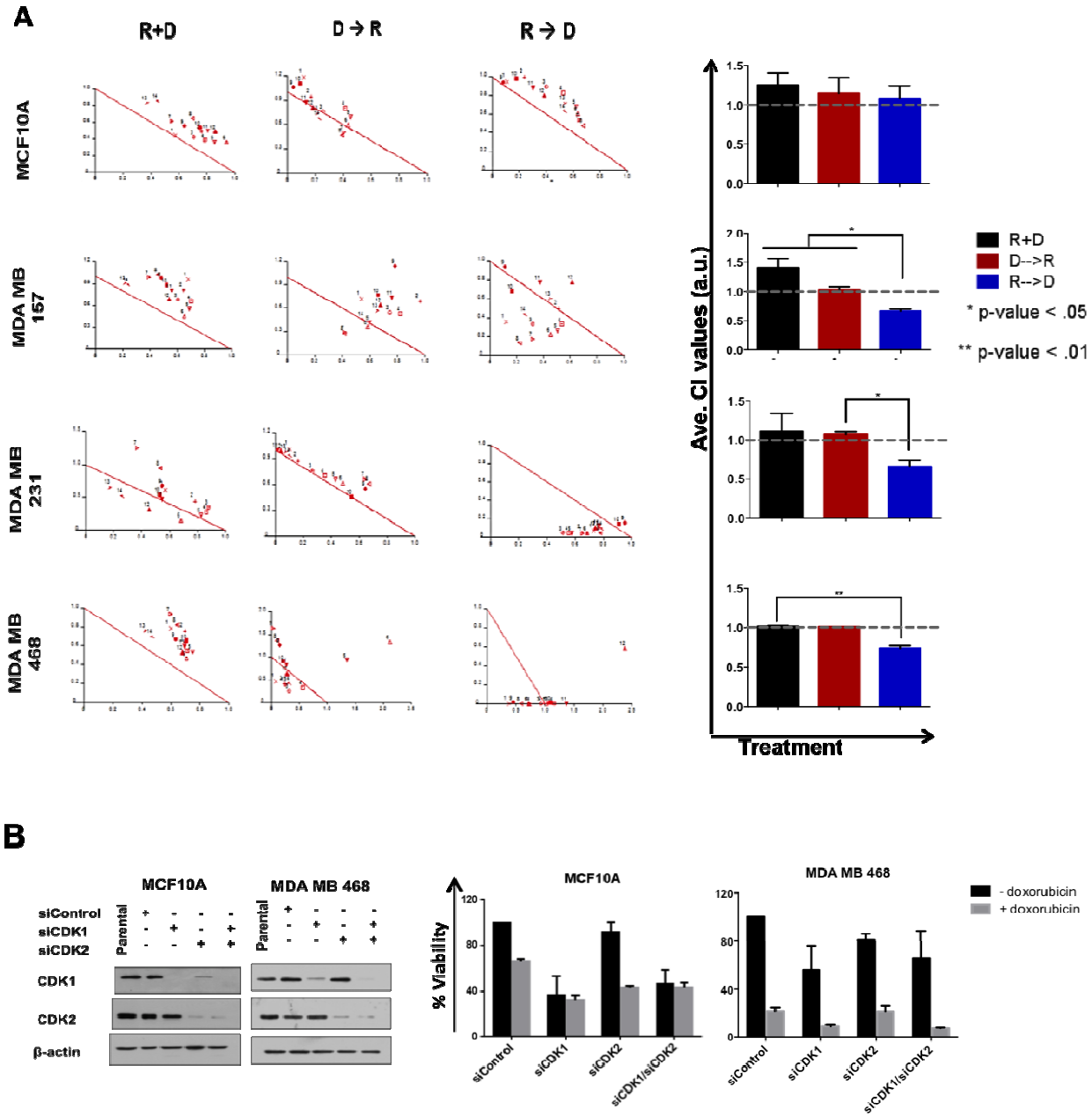


Figure 5. Sequential administration of roscovitine (R) and doxorubicin (D) induces synergistic cell inhibition only in TNBC cells. A, MCF10A and TNBC cells were treated with R and D simultaneously (R+D, left), D preceding R (D→R, middle) or R preceding D (R→ D, right). Isobolograms and CI values were generated using CalcuSyn. B, MCF10A and MDA MB 468 cells were transfected with either non-targeted siRNA (siControl) or siRNA targeting CDK1, CDK2 or both. Western blot analysis confirmed knockdown (left). Percent viability with and without doxorubicin treatment was assessed by MTT on day 12 (right). Mean ± SD.

2.3C COMBINATION TREATMENT INCREASES APOPTOSIS SPECIFICALLY IN TNBC CELLS

We used a luciferase caspase 3/7 assay to examine the apoptotic response of HMEC and TNBC cells to single and combination treatment. No increase in caspase 3/7 activities was detected in MCF10A cells. While doxorubicin and combination treatment did significantly increase caspase activity in TNBC cells compared to untreated cells (p-value < 0.05), there was no significant difference between the caspase activities of doxorubicin versus combination treated TNBC cells (Figure 6). These data suggested that both single and combination treatment induced apoptosis, but does not account for the synergistic cell inhibition measured only in TNBC cells

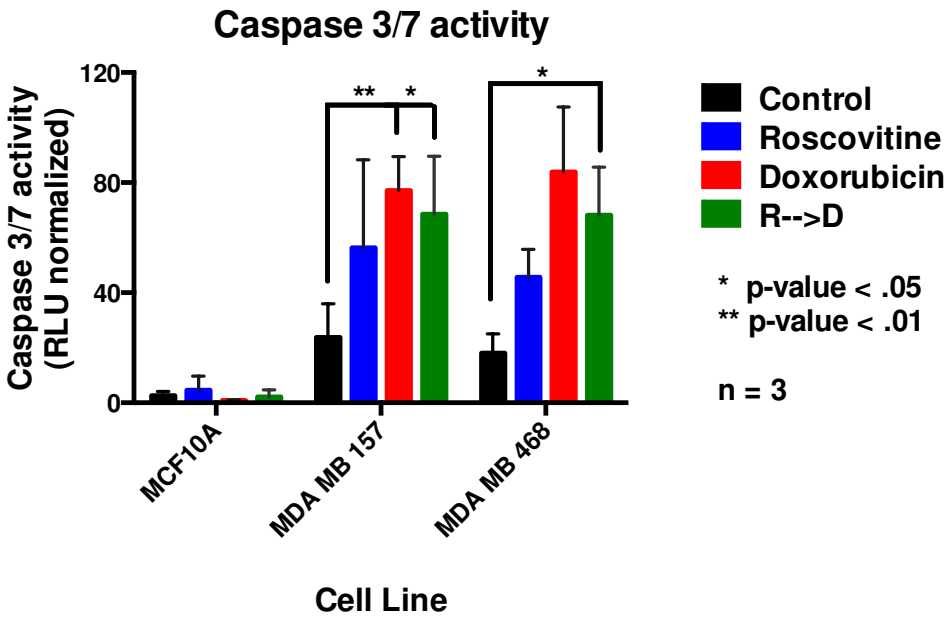


Figure 6. Doxorubicin and combination treatment induce caspase 3/7 activity. HMEC and TNBC cells were treated with single or combination drug treatment and subjected to a luciferase assay to measure caspase 3/7 activity.

Since caspase activity during drug treatment could not account for the synergistic response of combination treatment in TNBC cells, cell death was examined in TNBC and HMEC cells using a dye-exclusion assay in response to single and combination treatments both during and following drug treatment. Cells were subjected to a dye-exclusion assay with propidium iodide (PI). PI positivity is detected via flow cytometry when the cell membrane is compromised, a characteristic of apoptosis [353]. Roscovitine-induced PI positivity peaked at 24 hours post drug exposure (Figure 7A, left). Doxorubicin treatment steadily reduced cell viability over time; with MDA MB 157 cells showing over 30% PI positivity on day 12 (Figure 7A, right). When the drug was present, combination treatment induced 15% PI positivity in MCF10A cells, but induced 45% and 30% PI positivity in MDA MB 157 and MDA MB 468 cells, respectively (Figure 7, right). Moreover, only TNBC cells continued to exhibit decreased cell viability, or PI positivity, after release from treatment. MDA MB 157 cells had over 50% PI positivity 72 hours after being released from treatment, while MDA MB 468 cells had over 25% PI positivity 120 hours after being released from treatment (Figure 7A). These data demonstrate that the combination treatment inhibited TNBC cell recovery, causing the cells to continue to die after treatment and to a greater extent than HMEC cells.

Western blot analysis was performed to detect PARP-1 cleavage, a marker of apoptosis. Densitometry analysis was used to measure the ratio of cleaved PARP-1 to full length PARP-1. Staurosporine treatment, used as a positive control, induced PARP-1 cleavage in HMEC and TNBC cells (Figure 7B). MCF10A cells did not induce PARP-1 cleavage in response to treatment above basal levels. TNBC cells expressed cleaved PARP-1 both during and after release from combination treatment. MDA MB 157 cells had the highest ratio of PARP-1 cleavage at 48 hours into combination treatment, and maintained cleaved PARP-1 expression until 72 hours post release (Figure 7B). MDA MB 468 cells had the highest ratio of PARP cleavage 24 hours into combination treatment, and

also had a sustained apoptotic signal until 72 hours post release (Figure 7B). The persistence of cleaved PARP-1 expression, similar to the continuous PI positivity, indicates that TNBC cells continued to undergo apoptosis after treatment. These data demonstrate that combination treatment inhibits cell recovery, leading to a persistent apoptotic signal only in TNBC cells.

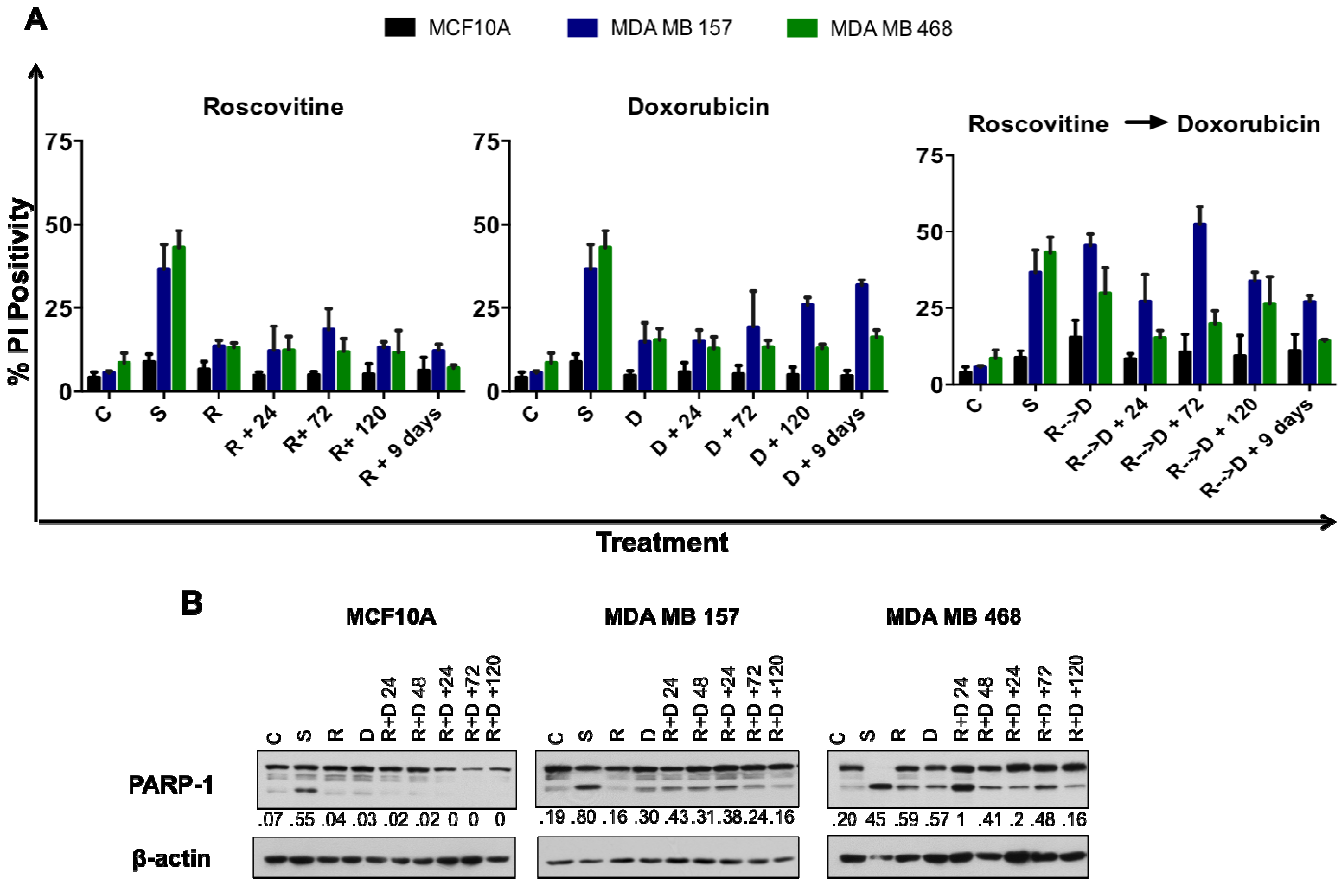


Figure 7. Combination treatment induces prolonged apoptosis only in TNBC cells. A, HMEC and TNBC cells were treated with roscovitine (R) for 24 hours, doxorubicin (D) for 48 hours, or roscovitine preceding doxorubicin (R→D) at IC_{50} concentrations. Staurosporine (S) treatment was used as a positive control. Cells were harvested at indicated times and subjected to a dye-exclusion assay using propidium iodide (PI). B, HMEC and TNBC cells were treated with R, D or R preceding D and harvested at indicated times. Western blot analysis was used to detect full-length and cleaved PARP1. Full PARP-1 and cleaved PARP-1 bands were quantified with Image J (ratio = cleaved/full PARP-1).

To determine if inhibition of CDK1, CDK2 or both is required for inducing apoptosis with doxorubicin, MCF10A and MDA MB 468 cells were transiently transfected with siRNA. Samples were harvested immediately after treatment and 48 hours post treatment. Cleaved PARP-1 expression was detected in all conditions in MCF10A cells immediately after treatment; however, the addition of doxorubicin to CDK knockdown did not increase cleaved PARP-1 expression. Indeed, simultaneous CDK1 and CDK2 knockdown plus doxorubicin had the least PARP cleavage in MCF10A cells (Figure 8A). In contrast, knockdown of CDK1, CDK2 or both increased PARP-1 cleavage in MDA MB 468 cells compared to siControl transfected cells. Moreover, the addition of doxorubicin to CDK knockdown increased cleaved PARP-1 expression (Figure 8C). Forty-eight hours post treatment, MCF10A knockdown and combination treated cells showed complete recovery, expressing only full-length PARP (Figure 8B). However, MDA MB 468 cells had persistent cleaved PARP1 expression in both transient knockdown cells and in cells that had CDK knockdown with doxorubicin 48 hours post treatment (Figure 8D). These findings reveal that TNBC cells cannot recover from potent CDK inhibition or CDK inhibition combined with doxorubicin, leading to an enduring apoptotic signal.

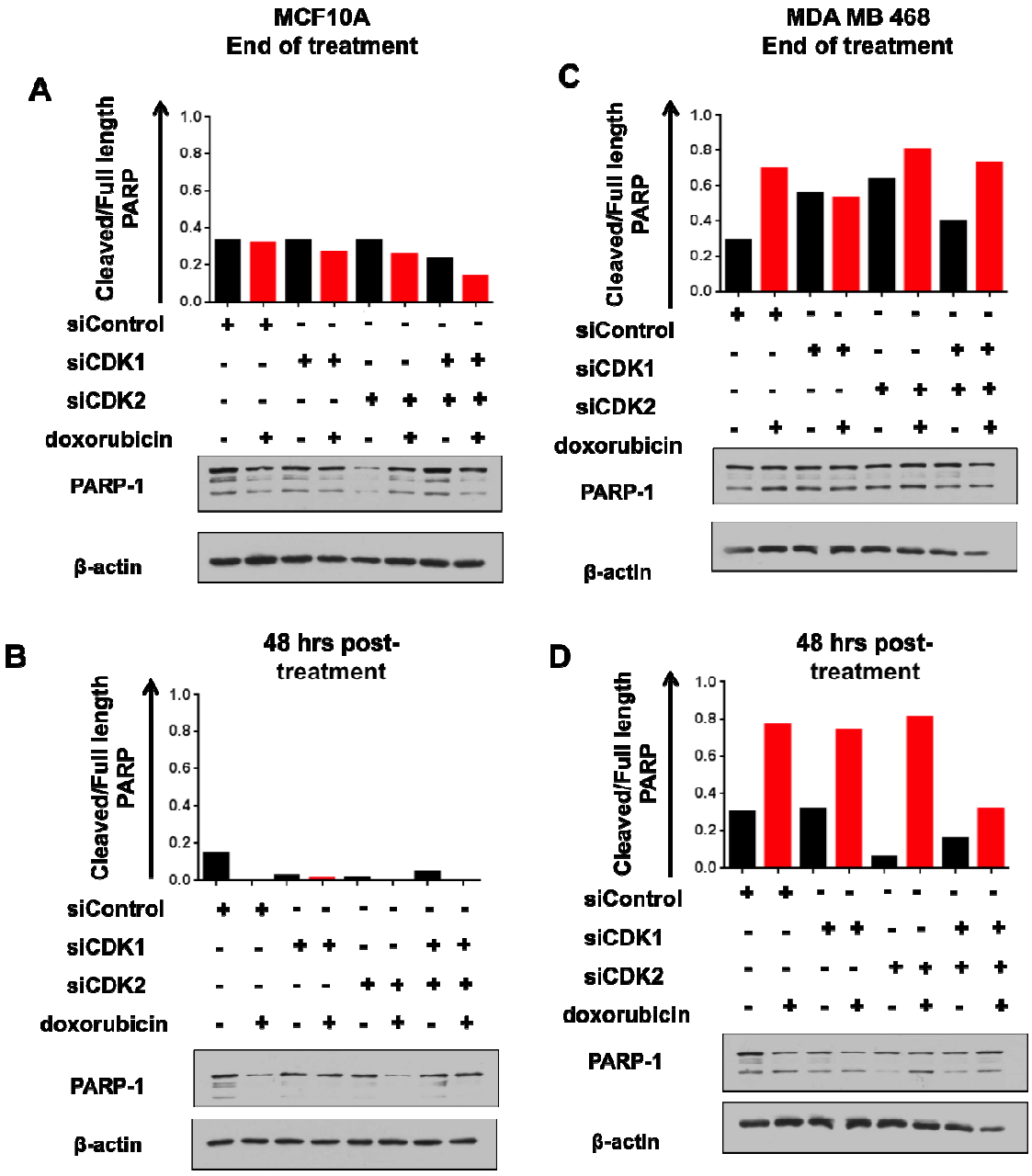


Figure 8. TNBC cells cannot recover from CDK inhibition plus doxorubicin. (A, B) MCF10A and (C, D) MDA MB 468 cells were transfected with siRNA with or without doxorubicin treatment. Cells were harvested directly after treatment (A, C) and 48 hours post treatment (B, D). Western blot analysis was used to detect PARP1. Full PARP and cleaved PARP bands were quantified with Image J (ratio = cleaved/full PARP).

2.3D ROSCOVITINE-DOXORUBICIN COMBINATION INHIBITS TUMOR GROWTH AND INCREASES SURVIVAL

To examine combination treatment in a pre-clinical model, MDA MB 468 cells were used to generate human xenograft tumors in nude mice. There were four treatment arms: vehicle, roscovitine (50 mg/kg) for 4 days on 3 days off, doxorubicin (2mg/kg) once a week and roscovitine (4 days) followed by 1 day of doxorubicin, 3 days off, all given for 4 cycles. Mice began receiving treatment when tumors reached a volume of 100-150 mm^3. Combination treated tumors did not increase in volume while on treatment, with tumors averaging at 125 mm^3 on day 26. Combination treated tumors were significantly smaller (p-value <0.01) than vehicle treated tumors that averaged 330mm^3 on day 26. Moreover, combination treated tumors were significantly smaller (p-value <0.05) than both roscovitine and doxorubicin treated tumors at the end of treatment (Figure 9 A, B). Following drug treatment, combination treated tumors remained significantly reduced compared to the other three treatment arms (Figure 9A). Notably, no measurable difference was observed between vehicle and roscovitine treated mice; supporting clinical findings that roscovitine is inefficient as a single agent.

None of the combination treated mice suffered from increased toxicity or tumor burden during the 60-day experiment. As such, combination therapy significantly increased overall survival (p-value < 0.05) compared to vehicle, roscovitine and doxorubicin treated mice (Figure 9C). It is imperative to ensure that novel combination therapies do not cause increased toxicity. Overall toxicity was assessed by weight loss during and following treatment. Based on weight loss, combination therapy did not increase toxicity (Figure 9D). Roscovitine treatment also did not cause weight loss toxicity (Figure 9D). However, 80% of doxorubicin treated mice had to be sacrificed due to >20% weight loss, again revealing the limitations of doxorubicin as monotherapy (Figure 9D, Table 15).

Western blot analysis of PARP-1 on tumors resected on day 26 found that combination therapy significantly increased apoptosis (p-value <0.05) compared to doxorubicin treated tumors. Also, p21 expression was not detected in any of the tumors (Figure 9E). Overall, this data suggests that roscovitine-doxorubicin combination therapy is both well tolerated and efficacious against TNBC, supporting future clinical studies.

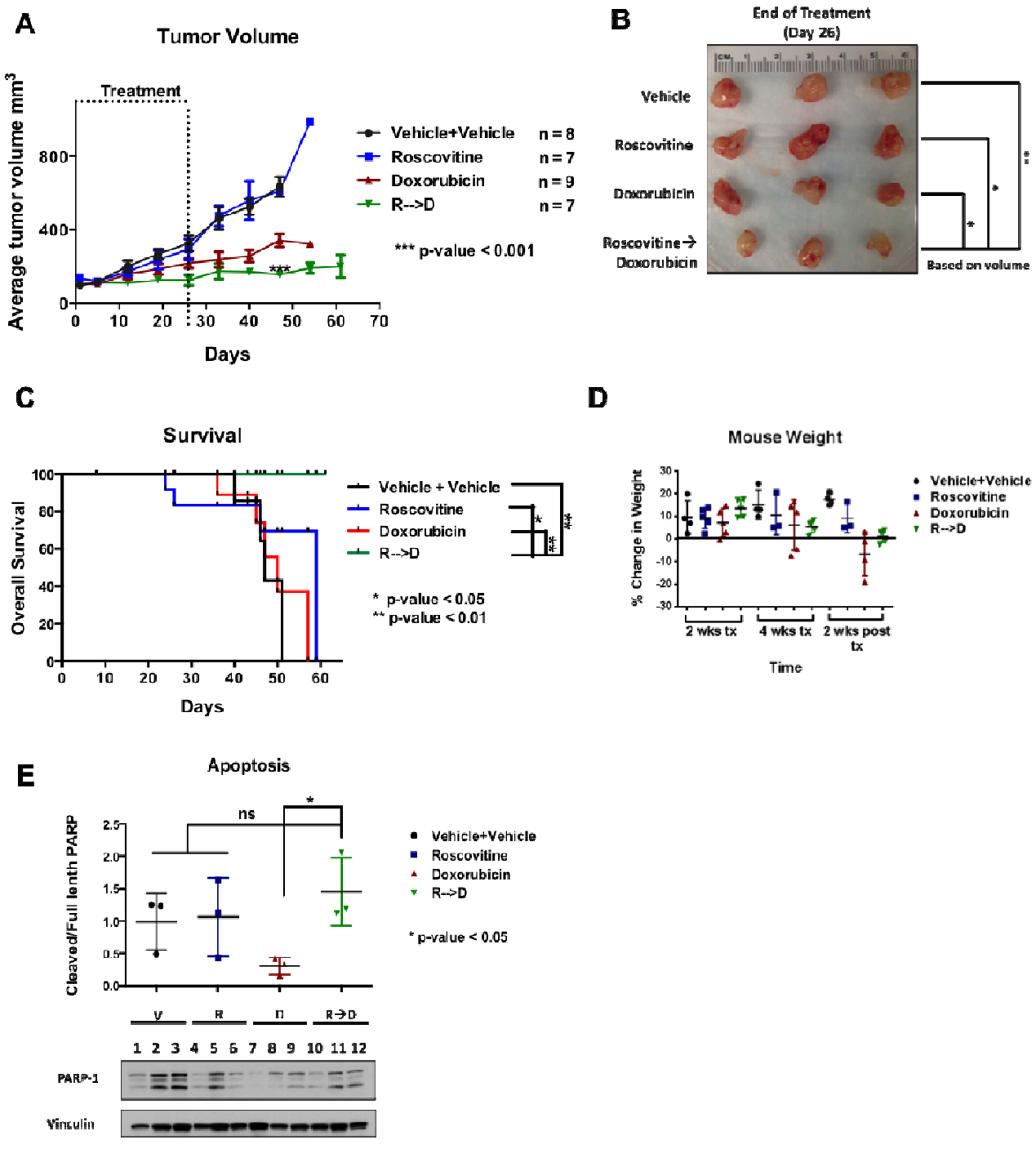

A
Tumor Volume
Treatment
Average tumor volume mm3
800
400
0
0 10 20 30 40 50 60 70
Days
Vehicle+Vehicle n = 8
Roscovitine n = 7
Doxorubicin n = 9
R-->D n = 7
*** p-value < 0.001
B
End of Treatment
(Day 26)
Vehicle
Roscovitine
Doxorubicin
Roscovitine→
Doxorubicin
Based on volume
C
Survival
Overall Survival
100
80
60
40
20
0
0 10 20 30 40 50 60
Days
Vehicle + Vehicle
Roscovitine
Doxorubicin
R-->D
* p-value < 0.05
** p-value < 0.01
D
Mouse Weight
% Change in Weight
30
20
10
0
-10
-20
-30
2 wks tx
4 wks tx
2 wks post tx
Time
Vehicle+Vehicle
Roscovitine
Doxorubicin
R-->D
E
Apoptosis
Cleaved/Full length PARP
2.5
2.0
1.5
1.0
0.5
0.0
ns
*
V
R
D
R→D
1 2 3 4 5 6 7 8 9 10 11 12
PARP-1
Vinculin
Vehicle+Vehicle
Roscovitine
Doxorubicin
R-->D
* p-value < 0.05

Figure 9. Combination treatment reduces tumor growth and increases overall survival without increasing toxicity. Mice were treated with vehicle, roscovitine (50 mg/kg 4 days on, 3 days off), doxorubicin (2mg/kg once a week), or roscovitine followed by doxorubicin for 4 cycles. A, Tumor volume (L x W^2 / 2) was measured twice a week. B, Representative tumors shown of the four treatment arms were resected at the end of treatment on day 26. Statistical analysis based on tumor volume. C, Kaplan-meier curve was generated to examine mouse overall survival. Survival analysis was performed using the Mantel-Cox test. D, Mouse weight was measured twice a week. Percent change from weight at start of treatment is shown at times indicated. E, Western blot analysis examining PARP-1 in protein lysates from tumors resected on day 26.

Table 15. Cause of euthanasia/death		
	Euthanasia due to tumor burden (%)	**Euthanasia due to weight loss/deteriorated health (%)**
Vehicle	100	0
Roscovitine	100	0
Doxorubicin	20	80
Roscovitine → Doxorubicin	0	0

2.4 CONCLUSIONS

Roscovitine treatment causes a G2/M arrest exclusively in TNBC cells that can be exploited to augment the doxorubicin-induced apoptotic response. The G2/M arrest detected only in TNBC cells suggests that the molecular characteristics that differentiate TNBC cells from non-TNBC cells can be exploited to target the cell cycle. Roscovitine also induced a G2/M arrest in HMEC 76NE6 cells, which share the TNBC cell characteristic of p53 inactivity. This similarity could be indicative of a marker for response, which will be thoroughly investigated in Chapter 3.

Combination treatment caused a sustained cell death signal specifically in TNBC cells. However, neither single or combination treatment caused a significant amount of apoptosis in HMEC cells. The prolonged combination-induced apoptotic response in TNBC cells inhibited cell recovery. Although roscovitine is a pan-CDK inhibitor, transient knockdown of CDK1 and CDK2 revealed that inhibition of CDK1 is sufficient to induce synergism with doxorubicin in TNBC cells. Moreover, the addition of doxorubicin to CDK knockdown cells also caused an increased apoptotic signal only in TNBC cells. These data validate the hypothesis that it is possible to induce synergistic cell death in TNBC cells while leaving non-malignant cells unharmed.

In vivo pre-clinical studies must be performed to translate a novel therapy to the clinic. Although both roscovitine and doxorubicin have been clinically examined, well-tolerated single agents can have increased toxicities when combined. For example, 27% of patients had cardiac dysfunction when treated with trastuzumab and an anthracycline [221]. However, our xenograft studies illustrate that combination treatment had increased efficacy over single agents without causing increased toxicity. The drugs were again administered sequentially. Other cancer models have corroborated the finding that the sequence of drug administration affects combination efficacy and toxicity. For example, a colorectal cancer pre-clinical model that examined the combination of the topoisomerase I inhibitor irinotecan

with 5-FU administered simultaneously and in both sequences found that when each drug was given at 50% MTD, irinotecan followed by 5-Fu caused the most growth inhibition without additional toxicity. However, when the drugs were given at 75%, the same sequence was 100% lethal [354]. These findings, along with our own studies, demonstrate that combination sequence and dosage is crucial for maximizing efficacy while limiting adverse effects, and will need to be closely monitored in the clinic.

The specificity of drug administration required for synergism also emphasizes that the effectiveness of combination treatment is pathway driven. The concept of synthetic lethality was born from genetic studies performed in drosophila that found that loss of one gene was tolerated due to overcompensation in another pathway. However, when genes from both pathways were inhibited, synthetic lethality occured [355]. This concept is now being applied to cancer therapy in order to maximize therapeutic benefit and limit toxicities [356]. PARP inhibitors, for instance, are synthetically lethal to cancer cells with BRCA mutations because these cells heavily rely on BER to compensate for impaired HR [170, 262]. Studies performed in non-mammalian systems have also yielded potential synthetic lethal therapies. Drug screenings done in yeast, a readily genetically modified system, found that yeast with mutations in DNA HR proteins were especially sensitive to cisplatin [357]. These findings illustrate the benefit of using DNA damage agents in cancer cells with altered or impaired DNA repair pathways to establish a synthetic lethality.

The roscovitine-doxorubicin combination can be considered synthetic lethal in TNBC cells because it targets two major deregulated pathways in tumorgenesis, cell cycle regulation and DNA damage response. Sporadic TNBC tumors are often characterized as having a “BRCAness” phenotype, or have BRCA1/BRCA2 dysfunction or downregulation, which is associated with deregulated HR and sensitivity to anthracycline-and platinum-based chemotherapies [242, 358-361]. While the cell lines used in this study are BRCA wildtype, roscovitine has been shown to impair HR, one of the pathways that would be

required to repair doxorubicin-induced DNA damage [362]. Cancer cells often upregulate DNA repair pathways to survive assault from DNA-damaging agents used in therapy [356]. Therefore, by potentially inhibiting DNA repair with roscovitine while targeting anthracycline-sensitive TNBC cells with doxorubicin, we have generated a synthetic lethal combination. The proceeding chapter will further elucidate the possible mechanism of TNBC sensitivity to roscovitine-doxorubicin combination treatment.

CHAPTER 3: ROSCOVITINE-DOXORUBICIN SYNTHETIC LETHALITY REQUIRES P53 PATHWAY ABLATION

3.1A EFFECT OF CHECKPOINT DEREGULATION ON THERAPEUTIC RESPONSE

Mutation or deregulation of checkpoint proteins Rb and p53 has been shown to affect prognosis and response of cancer cells to therapy. Rb pathway deregulation can occur through multiple mechanisms, including Rb gene mutations, cyclin D1 amplification/overexpression, CDK4 overexpression or point mutations, gene deletions or epigenetic silencing of CKIs p16 and p14 (Table 2). Rb deregulation is often associated with poor prognosis [363]. Loss of Rb heterozygosity was measured in 72% and 62% of the more aggressive BLBC and Luminal B breast cancer subtypes, respectively [364]. However, the effect of deregulation of the Rb pathway on prognosis and therapeutic response is subtype-specific. In patients with ER-positive tumors, the Rb-loss gene signature correlated with decreased relapse free survival [363]. Notably, ablation of the Rb pathway can reduce sensitivity to endocrine therapy and is associated with tamoxifen resistance [365]. However, for patients with ER-negative tumors, the Rb-loss signature was associated with improved relapse-free survival [363]. Disruption of the Rb pathway also correlated to increased sensitivity to cisplatin and ionizing radiation in cell culture and in xenograft models [365]. In accordance with these findings, an Rb-loss signature was associated with increased pCR in both ER-positive and ER-negative breast cancer patients who received neoadjuvant 5-FU/doxorubicin/cytoxan and taxane/doxorubicin chemotherapy regiments [366]. The paradox of Rb-loss being attributed to more aggressive tumors, but leading to increased sensitivity to DNA damaging agents, may be because cells that lack a G1 checkpoint are more sensitive to chemotherapy [367, 368].

The effect of p53 mutations on prognosis and treatment response is less straightforward. Similar to Rb, p53 is subject to a variety of gene mutations in cancer including, missense or point mutations, gene amplification of the p53 inhibitor MDM2, GOF

mutations and inhibition from viral oncogenic proteins (Table 2) [90]. The impact of compromised p53 function on treatment response may be dependent on the type of mutation present [369]. Examination of 60 cell lines, including breast, prostate, lung, colon, kidney, ovarian cancer, leukemia and melanoma cell lines, against 123 anti-cancer agents found that p53 mutant cell lines tended to be more resistant to the growth inhibitory effects of most compounds, including DNA cross-linking agents, antimetabolites and topoisomerase I and II inhibitors, compared to p53 wildtype cell lines. However, response to antimitotic agents was independent of p53 status, indicating that the effect of p53 mutations on treatment may also be dependent upon the agent used [370]. IHC analysis of p53 expression in tumors from NSCLC patients after receiving treatment found that aberrant overexpression of p53 correlated to cisplatin resistance and reduced pathological response [371]. However, disruption of p53 function via transfection of viral oncoprotein HPV-E6 or expression of a dominant negative p53 mutant increased sensitivity of ER-positive breast cancer cells MCF7 to cisplatin treatment *in vitro*. Here, it was hypothesized that G1 checkpoint deregulation and/or reduced NER contributed to increased cisplatin-sensitivity in MCF7 p53 mutant breast cells [372]. Clinical studies found that mutations in the zinc binding L2 and L3 domains of p53 (codons 163-195 and 236-251, respectively), which interact with DNA, were associated with decreased survival and de novo resistance to doxorubicin monotherapy in breast cancer patients [373, 374]. Moreover, meta-analysis of 2,319 breast cancer patients found that when using a relative hazard ratio (RH), in which RH>1 indicates poor prognosis, node-negative and node-positive patients who had p53 gene alterations had an RH of 1.7 and 2.6, respectively. The RH >1 in both sets of patients associated p53 mutations with more aggressive breast tumors [375]. Also, microarray analysis of p53 wildtype and p53 mutant breast cancer tumors identified a 32-gene p53 signature. The study found that 89% of ER-negative tumors and 79% of grade III tumors were classified into the p53-mutant cluster. Moreover the p53 gene-signature correlated

with decreased disease free survival both in ER-positive breast cancer patients who received adjuvant tamoxifen treatment and in ER-negative patients. Similarly, the gene signature predicted that patients from the Sorlie, *et al*. study that received doxorubicin or 5-FU and mitomycin and had tumors with p53-mutant like gene expression had a 35% probability of 5-year survival compared to a 90% probability for patients with p53-wildtype like expression [176, 376]. However, there was a significant correlation with premenopausal breast cancer patients who had p53 overexpression (IHC) and response to treatment with cyclophosphamide/methotrexate/5-FU [377]. Therefore, although there are some inconsistencies in the literature on the effect of p53 dysfunction, p53 mutations are generally correlated with more aggressive tumors that are refractory to treatment.

3.1B CYCLIN DEPENDENT KINASES PARTICIPATE IN DNA DSB REPAIR

Several DSB DNA repair proteins are substrates for CDKs. CDK phosphorylation can affect function and complex formation of DNA repair proteins. Notably, CDK activity can serve as a molecular switch between faithful DNA repair and more error-prone pathways (Table 16).

Table 16. CDKs participate in DNA DSB repair

CDK	Substrate (Site)	Function	Ref.
CDK1	53BP1 (Unknown)	Unknown, however, yeast studies indicate a role in checkpoint activation through Chk1	[378, 379]
	CtIP (T847)	Required for DNA end resection	[113]
	BRCA2 (S3291)	Inhibits binding to Rad51	[380]
Unknown (CDK1 or CDK2)	CtIP (S327)	Required for CtIP/BRCA1/MRN complex formation. Could serve as a switch between HR and MMEJ	[114, 381, 382]

As previously described, the MRN complex identifies DSBs for repair. The ATM substrate p53 binding protein 1 (p53BP1) also accumulates at the site of damage to facilitate recruitment of downstream repair proteins, such as BRCA1, and is required for accurate intra-S phase and G2/M checkpoint activation [378, 383]. The CDK1-cyclin B complex has been shown to phosphorylate 53BP1 both *in vitro* and *in vivo* in proliferating mammalian cells. Moreover, treatment with roscovitine inhibited CDK1 phosphorylation of 53BP1 [379]. While the functional role of CDK1 phosphorylation of 53BP1 is currently unknown in mammalian cells, yeast protein Cdc2 (CDK1 homologue) phosphorylates the 53BP1 orthologue Crb2 at T215 following DNA damage, leading to checkpoint activation through Chk1 regulation [384, 385]. Cdc2 phosphorylation stimulates binding of the DNA repair protein Cut5 (orthologue to human TopB1), which promotes Crb2 accumulation at the DNA damage site and is required for Chk1 activation [385]. Further investigation is needed to confirm the role of 53BP1 in activating Chk1 upon DNA damage in mammalian cells.

Following initial recognition of the damage site, to undergo HR, DNA end resection is required at the damage site to generate single strands (Figure 2). Yeast studies demonstrated that CDK1 phosphorylates the endonuclease Sae2 at Ser267, which is required for DNA end resection [112, 386]. Moreover, mutating the Ser267 site on Sae2 to prevent CDK phosphorylation resulted in hypersensitivity to the topoisomerase I inhibitor camptothecin, indicating that CDK activity is necessary for repair of DSBs [386]. The human orthologue of Sae2 is CtIP, which is recruited to DNA DSBs only during S and G2 for end resection [387]. CDK1 also phosphorylates CtIP at Thr847. Treatment of U2OS cells with roscovitine inhibited phosphorylation at the Thr847 site. Furthermore, U2OS cells with a T847A mutation, which prevents CDK1 phosphorylation, were hypersensitive to camptothecin. U2OS mutant cells that mimicked CtIP constitutive activation with a T847E mutation were resistant to camptothecin treatment. Quantification of γ-H2AX revealed that camptothecin caused similar amounts of DNA damage in CtIP mutants; however, T847A

mutants had a significant reduction of RPA recruitment, which is downstream in the DNA end resection pathway (Figure 2) [113]. The observation that CDK1 activity was required for recruitment of downstream repair proteins, such as RPA, was corroborated in studies that found that roscovitine treatment reduced the ability of sarcoma cells to recruit RPA when exposed to ionizing radiation despite the formation of γ-H2AX foci [362]. Therefore, CDK activity is imperative for end resection and the recruitment of downstream repair proteins during HR.

CDK phosphorylation of CtIP is also important for DNA repair complex formation. CtIP has an additional CDK phosphorylation site at Ser327, which is required for its interaction with BRCA1 and MRN [381, 388]. The CtIP/MRN/BRCA1 complex occurs during S and G2, with roscovitine treatment abrogating the IR-induced interaction between BRCA1 and MRN [381]. Moreover, CDK phosphorylation of CtIP at Ser327 may serve as a switch between more accurate pathways of DNA repair (e.g. HR) and a recently discovered process called microhomology-mediated end joining (MMEJ). MMEJ occurs during G1 of the cell cycle phase and can repair DSBs in an error-prone manner that can contribute to genomic instability [114]. MMEJ repairs DNA DSBs differently from NHEJ in that it involves end resection followed by ligation of complimentary ends of DNA, resulting in nucleotide deletions [382]. An avian B-cell line DT40 model system demonstrated that while HR is dependent upon CDK phosphorylation at Ser327, MMEJ is not. However, CtIP may still provide the endonuclease activity required for MMEJ. This suggests that inhibition of CDK activity can lead to a more error-prone method of DNA repair of DSBs, with CDK phosphorylation of CtIP serving as a switch between HR and MMEJ [114].

Paradoxically, CDK phosphorylation of the HR protein BRCA2 can inhibit its activity [380]. BRAC2 facilitates loading of Rad51 onto the single strand filaments during HR (Figure 2) [117]. BRCA2 has a CDK phosphorylation site at Ser3291 at its C-terminus. CDK phosphorylation at Ser3291 prevents BRCA2 binding to Rad51 and is low during S-

phase, but increases as cells enter mitosis during normal cell cycle progression. Due to the increase in phosphorylation of BRCA2 specifically in nocodazole treated cells, which arrests cells in mitosis, the CDK1-cyclin B complex is most likely responsible for BRCA2 phosphorylation. However, both CDK1-cyclin A and CDK1-cyclin B complexes could phosphorylate BRCA2 in *in vitro* kinase assays. Exposure to IR reduced CDK kinase activity and phosphorylation of BRCA2. Therefore, CDK phosphorylation impairs the BRCA2-Rad51 interaction unless DNA DSBs are detected [380].

Overall, CDK activity has an important role in the recruitment and regulation of repair proteins required for DNA DSBs. While necessary for checkpoint activation, complex formation and DNA end resection, CDK activity must eventually be reduced in order for HR to occur. Importantly, CDK activity ensures a more accurate mode of DSB repair. The absence of CDK activity can lead to a reliance on MMEJ for DSB repair, which can cause gene deletions and tumor-promoting chromosome rearrangements [114, 389]. Thus, inhibiting CDKs could potentially compromise DNA repair, sensitizing tumor cells to DNA-damaging agents.

3.1C HYPOTHESIS AND SPECIFIC AIMS

Although the effect of G1 checkpoint deregulation on endocrine therapy and chemotherapy has been examined, it is unknown what effect Rb and p53 pathway ablation will have on combination treatment response. Moreover, CDK inhibition consistently compromises HR, possibly forcing cells to rely on more error-prone DDR pathways and increasing genomic instability. Therefore, *we hypothesized that G1 checkpoint ablation is required for roscovitine-doxorubicin-induced synergism in TNBC cells*. This hypothesis was addressed with the following specific aims:

- Examine the effect of combination treatment on the cell cycle in HMEC and TNBC cells.

- Investigate the effect of combination treatment on G1 checkpoint pathways in HMEC and TNBC cells.
- Determine how ablation of Rb and p53 pathways affects response to combination treatment.
- Elucidate the mechanism that predisposes TNBC cells to roscovitine-doxorubicin combination sensitivity.
- Examine the effect of combination treatment on DNA damage response and repair in HMEC and TNBC cells.

The data presented in this chapter shows that TNBC cells maintain a G2/M arrest in response to doxorubicin and sequential roscovitine-doxorubicin treatment, while HMEC cells are able to activate their G1 checkpoint in response to treatment. Furthermore, ablation of the p53 pathway, and not the Rb, is crucial to roscovitine-doxorubicin induced synergism. Detection of DNA repair foci demonstrate that combination treatment increases DNA damage explicitly in TNBC cells, while simultaneously reducing the ability of these cells to recruit downstream HR repair proteins despite being arrested in the G2/M cell cycle phase.

3.2 METHODS AND MATERIALS

3.2B CELL CULTURE

For medium ingredients and cell line origin of 76NE6, 76NF2V, MCF10A, MDA MB 157, MDA MB 231 and MDA MB 468 please see chapter 2.2A. The colorectal cancer cells HCT116 p53 wildtype ($p53^{+/+}$) and p53 knockout ($p53^{-/-}$) were obtained from Dr. Junjie Chen and maintained in DMEM supplemented with 10% FCS. HEK-293T cells for lentiviral packaging were maintained in DMEM supplemented with 10% FCS.

3.2C DRUGS

For origin and dilution of roscovitine and doxorubicin please see chapter 2.2B. Etoposide (Sigma-Aldrich) was diluted in DMSO to 10 mM and used at 5 μM.

3.2D FLOW CYTOMETRY

Cells were seeded in 10 cm dishes at 3.5 x 10^5 cells per plate to examine cell-cycle phases in response to single and combination drug treatment. After 24 hours, cells were treated with roscovitine for 24 hours, doxorubicin for 48 hours or roscovitine followed by doxorubicin at IC_{50} concentrations (Table 14). Cells were harvested after treatment and 24 hours-post treatment as indicated. For methods of cell harvesting and sample analysis please see chapter 2.3C.

3.2E HIGH-THROUGHPUT SURVIVAL ASSAY

For procedures on HTSA, including method of drug administration, harvesting with MTT and cell inhibition analysis, please see chapter 2.2D. Number of cells seeded and IC concentrations of roscovitine and doxorubicin are found in Tables 17 and 18, respectively.

Table 17. Cell number/well seeded for HTSA

Cell Line	Cells/well
76NE6	150
76NF2V	1000
HCT116 ($p53^{+/+}$ and $p53^{-/-}$)	750

Table 18. Drug concentrations used for combination treatment (R→D)

Cell Line	R (μM) ($IC_{10, 25, 50}$)	D (nM) (IC_{10}-IC_{50})
76NE6	3, 6, 9	1-14
76NF2V	15, 20, 22	1-14
HCT116 ($p53^{+/+}$ and $p53^{-/-}$)	6, 12, 20	5-35

3.2F WESTERN BLOT ANALYSIS

For methods on protein lysate extraction, western blot analysis or protein detection, please see chapter 2.2G. Antibodies against PARP-1, CDK1, total Rb and phospho-Rb

(Ser807/811) (Cell Signaling Technology, Beverly, MA), CDK2 (Santa Cruz, Dallas, TX), p27 (BD Biosciences, San Jose, CA), p53, p21, and actin (EMD Millipore, Billerica, MA) were used to probe for protein expression.

3.2G GENERATION OF STABLE KNOCKDOWN

To generate Rb stable knockdown in 76NF2V cells, HEK-293T cells were transfected with lentiviral packaging vectors pMDG.2 and pCMV deltaR8.2 (produced by the Didier Trono laboratory (Lausanne, Switzerland) and made available through the Addgene repository) and lentiviral vector containing shRNA against Rb or scramble sequence (University of Texas MD Anderson Cancer Center ShRNA and ORFeome Core Facility) using LipoD293 transfection reagent (SignaGen, Rockville, MD) according to manufacturer's protocols. Virus-containing medium was filtered through 0.45 µM filters and directly added to target cells in the presence of 8 µg/ml polybrene (Millipore). 76NF2V cells were selected in 2 µg/ml puromycin (InvivoGen, San Diego, CA) and maintained at half selection concentration. Knockdown of Rb was confirmed via Western blot analysis.

3.2H TRANSFECTION

For the protocol used to transfect siRNA against CDK1, CDK2 or both CDKs in HMEC and TNBC cells, please see chapter 2.2H

3.2I RT-qPCR

To measure transcription of p21, cells were subjected to roscovitine for 24 hours, doxorubicin for 48 hours or both drugs sequentially at IC_{50} concentrations, with untreated cells serving as a negative control (Table 14). Twenty-four hours of etoposide at 5 µM treatment served as a positive control. Following drug treatment, cells were harvested and RNA was extracted using the Qiagen RNeasy Mini Kit according to manufacturer's protocols (Venlo, Netherlands). Cell extracts were DNAase treated (Qiagen). Following RNA extraction, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (AB Biosystems, Foster City, CA) according to manufacturer's protocols. Gene transcription

of p21 was measured using SYBR Green Jumpstart Taq Ready Mix (Sigma-Aldrich) on a 7500 Fast Real-Time PCR System equipped with 7500 software version 2.3 (AB Biosystems). Forward primer used for p21 was (5'-3'): ACTCTCAGGGTCGAAAACGG and reverse primer was (5'-3'): CCTCGCGCTTCCAGGACTG (Sigma-Aldrich). Relative mRNA was normalized to GAPDH.

3.2J NEUTRAL COMET ASSAY

A neutral comet assay was performed according to Trevigen (Gaithersburg, MD) protocols to measure DNA double-strand breaks (DSBs) in HMEC and TNBC cells in response to single and combination drug treatment. Briefly, following treatment with single or combination drug treatment, cells were harvested from 10 cm dishes, combined with low-melting agarose, and spread onto CometSlides (Trevigen). After allowing the cells to adhere to the slides, cells were lysed with Trevigen lysis solution. The slides were then placed in an electrophoresis chamber with neutral electrophoresis buffer. A current of 21 V was used for 21 minutes. After drying, samples were stained with SYBR Green I and allowed to dry at room temperature in the dark. Images of nuclei were captured using an Eclipse 90i microscope equipped with the NIS-Elements Br 3.10 software program (Nikon, Tokyo, Japan). The tail moment was measured using the CometScore software program (TriTek, Sumerduck, VA).

3.3K IMMUNOFLUORESCENCE TO DETECT DNA REPAIR FOCI

For γ-H2AX and Rad 51 immunofluorescence, HMEC, TNBC and HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were seeded on 8-well chamber slides (Thermoscientific, Rochester, NY) and treated with roscovitine for 24 hours, doxorubicin for 48 hours or both drugs sequentially all at IC_{50} concentrations (Table 14). Cells were fixed in 4% paraformaldehyde for 20 minutes followed by permeabilization with a 0.3% triton solution (20mM Hepes, 50mM NaCl, 3mM MgCl2, 300mM sucrose and TritonX-100) for 20 minutes. Cells were blocked in PBS with 10% BSA and 2% horse serum for one hour. Antibodies were diluted 1:500 and 1:1000

for anti-γ-H2AX (EMD Millipore) and anti-Rad51 (generously provided by Dr. Junjie Chen), respectively, and incubated at 4℃ overnight. Secondary goat anti-mouse or goat anti-rabbit antibodies (Alexa Fluor 594 and 488, respectively, EMD Millipore) were diluted at 1:750 and incubated at room temperature at 1 hour. Nuclei were stained with DAPI (Life Technology, Grand Island, NY) at 1μg/ml for 5 minutes at room temperature. Cells were mounted with Dako fluorescent mounting medium (Carpentaria, CA). Images for quantification were captured using the Olympus FV1000 Laser Confocal Microscope at 60X magnification (Tokyo, Japan). Images for corresponding figures were captured using Eclipse 90i microscope equipped with the NIS-Elements Br 3.10 software program at 100X magnification. At least 100 cells were counted per sample per trial. Cells with ≥5 foci of γ-H2AX were considered positive. Cells with ≥1 foci of Rad51 were considered positive.

3.2L STATISTICAL ANALYSIS

The Student *t*-test with a 95% confidence interval was performed to determine p values. P values less than 0.05 were considered significant. Mean ± SD.

3.3 RESULTS

3.3A SINGLE AND COMBINATION DRUG TREATMENT MAINTAIN A G2/M ARREST ONLY IN TNBC CELLS

To determine the effect of single and combination treatment on the cell cycle, TNBC and HMEC cells were treated with roscovitine (24 hours), doxorubicin (48 hours), or sequential roscovitine-doxorubicin (cumulative 72 hours) at IC_{50} concentrations followed by cell-cycle-phase FACS analysis. As seen in Figure 10, single and combination treatment had little effect on the cell-cycle-phase profile of MCF10A cells, with over 70% cells continuing to remain in G1. However, doxorubicin treatment induced over 20% polyploidy in the TNBC cell line MDA MB 231, an effect that was maintained during combination treatment (Figure 10A). Notably, following a 24-hour release from doxorubicin and

combination treatment, 45% and 30% of MDA MB 231 cells had polyploid nuclei, respectively. Doxorubicin reduced G1 phase cells in MDA MB 157 cells by 10% compared to control. This G1 phase reduction was maintained in combination treatment, with nearly 30% cells entering polyploidy (Figure 10B). Fifty-percent of MDA MB 468 cells accumulated in G2/M in response to doxorubicin and combination based treatment (Figure 10B). Unlike HMEC cells that accumulated in G1, accumulation of TNBC cells in G2/M and/or induction of polyploidy in response to combination treatment suggests that TNBC cells do not have an intact G1 cell cycle checkpoint.

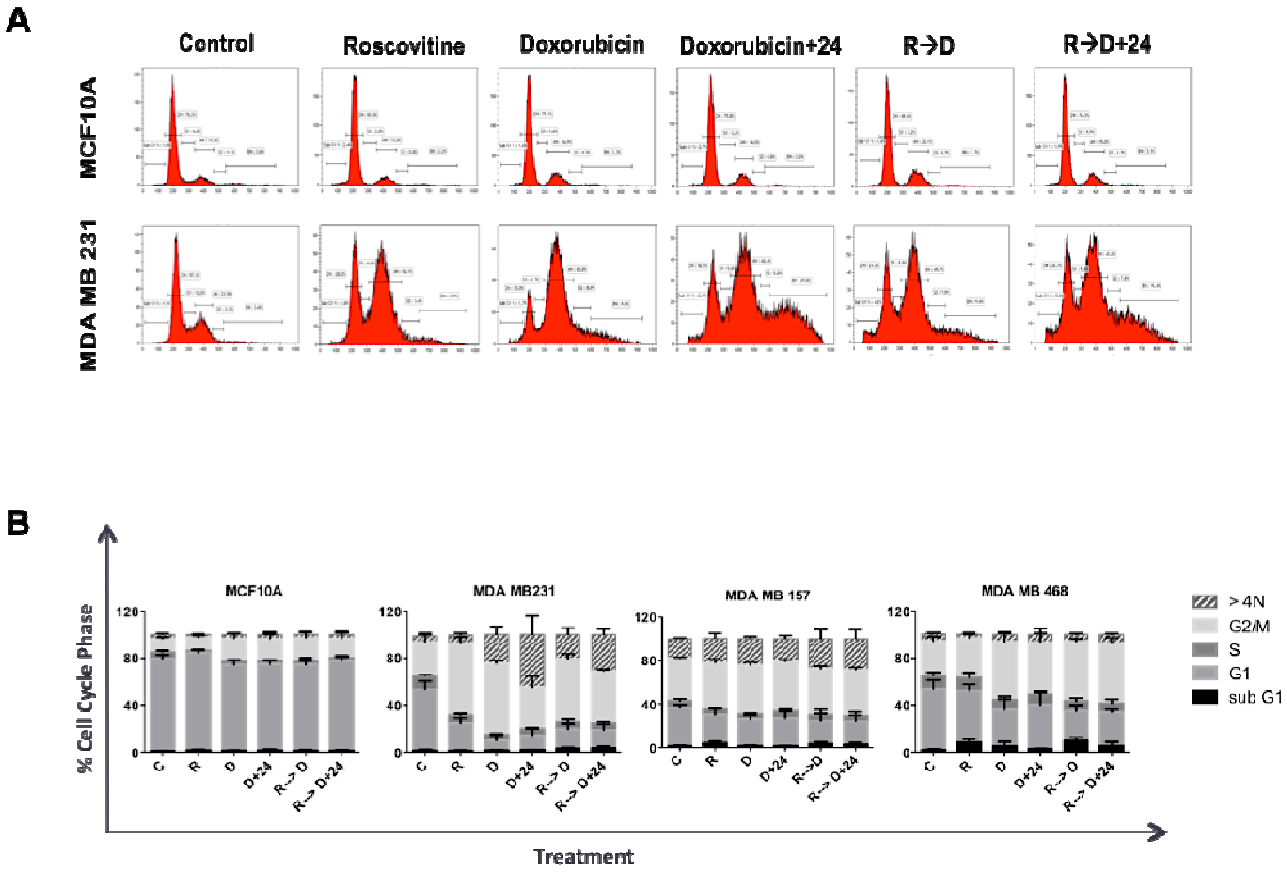


Figure 10. Single and combination drug treatment induces a G2/M arrest and polyploidy only in TNBC cells. A, MCF10A and MDA MB 231 cells were treated with roscovitine, doxorubicin or roscovitine followed by doxorubicin and subjected to cell cycle analysis via flow cytometry. Graphs, generated using Kaluza, are representative of three trials. B, HMEC and TNBC cells were treated with single and combination treatment followed by flow cytometry. Three trials were performed per cell line.

3.3B KNOCKDOWN OF CDK1 IS SUFFICIENT TO AUGMENT G2/M ARREST WITH DOXORUBICIN

To determine if inhibition of CDK1, CDK2 or both is required for the G2/M arrest and induction of polyploidy in TNBC cells, we transiently transfected HMEC and TNBC cells with siRNA with and without the addition of doxorubicin (IC_{50} concentration) followed by cell cycle analysis. Western blot analysis of CDK1 and CDK2 confirmed knockdown (Figure 11A). Cell cycle analysis revealed that MCF10A cells only had about a 10% increase in G2/M upon CDK knockdown, with the addition of doxorubicin either reverting cells to a G1 accumulation (70-80%) or causing no change (Figure 11B). In TNBC cells, knockdown of CDK1 or CDK1/CDK2 lead to a G2/M accumulation that was augmented with the addition of doxorubicin. Indeed, 80% of MDA MB 468 cells accumulated in the G2/M cell cycle phase when knockdown of CDK1 was combined with doxorubicin treatment, demonstrating a 20% or 40% increase in G2/M cells compared to knockdown or doxorubicin treatment alone, respectively. There was no additional gain in the G2/M phase when both CDKs were knocked down in the presence of doxorubicin compared to CDK1 knockdown plus doxorubicin in TNBC cells (Figure 11B). Therefore, while HMEC cells arrest in G1 in response to CDK inhibition and/or doxorubicin treatment, TNBC cells (with p53 mutations and Rb pathway inactivation) may lack the ability to arrest in G1, forcing them to accumulate in G2/M.

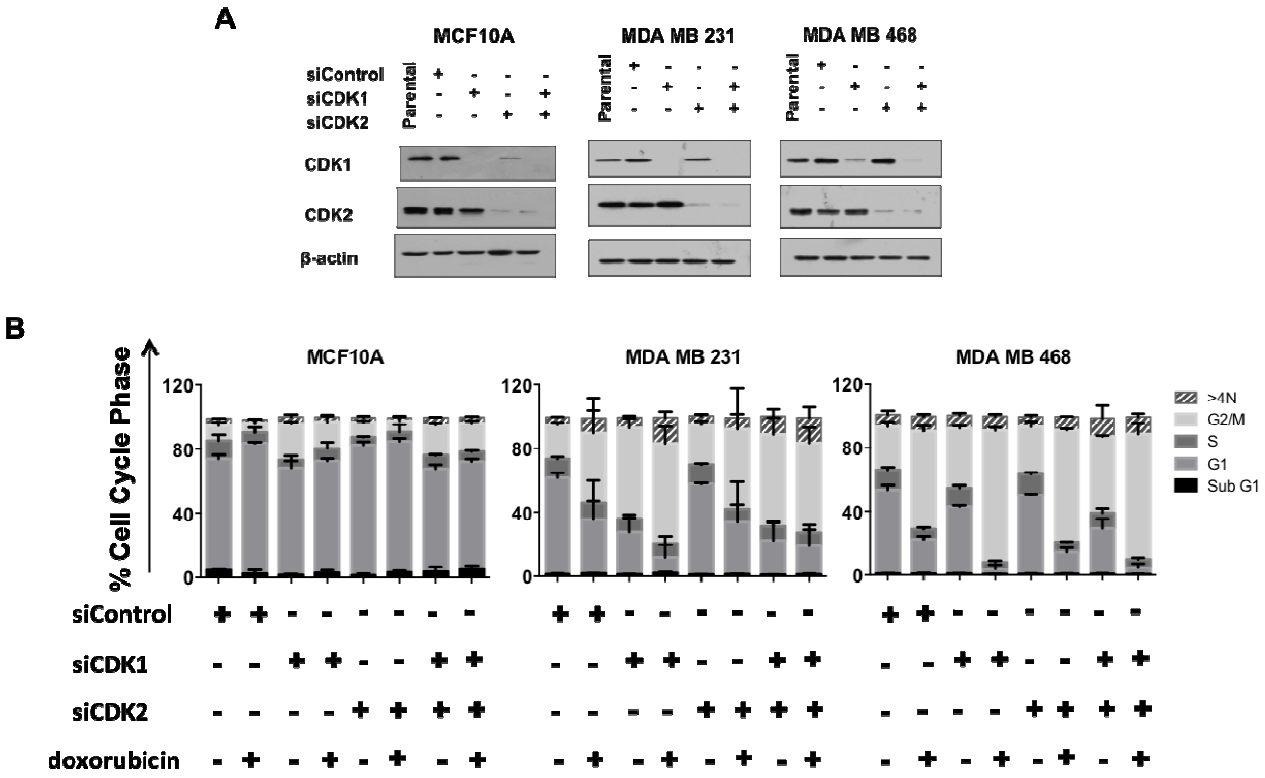


Figure 11. Knockdown of CDK1 is sufficient to induce a G2/M arrest in the presence of doxorubicin. A, HMEC and TNBC cells were transiently transfected with siRNA against CDK1, CDK2 or both. Non-targeting siControl was used as a negative control. Western blot analysis confirmed knockdown. B, Following knockdown with and without doxorubicin treatment, HMEC and TNBC cells were subjected to cell cycle analysis via flow cytometry.

3.3C SINGLE AND COMBINATION DRUG TREATMENT INDUCE G1 CHECKPOINT ARREST ONLY IN HMEC CELLS

Activation of G1 checkpoint proteins was examined in both HMEC and TNBC cells through Western blot analysis and RT-qPCR. MCF10A cells showed reduced expression of phospho-Rb in response to doxorubicin and combination treatment, suggesting Rb is actively inhibiting transcription factor E2F and inducing G1 checkpoint activation. Doxorubicin and combination treatment also increased p27 expression in MCF10A cells (Figure 12A). Additionally, MCF10A cells had p53 pathway activation as measured by p21 expression. MCF10A cells had a modest increase in both p21 protein expression and transcription in response to treatment, with combination treatment inducing a 3-fold increase in p21 transcription (Figure 12A, B). Etoposide treatment induced over a 15-fold increase in p21 transcription, exemplifying the ability of HMEC cells to induce p53 activity upon DNA damage.

In contrast, drug treatment increased phospho-Rb expression in TNBC MDA MB 157 cells, indicating Rb inhibition (Figure 12A). Furthermore, MDA MB 157 cells showed no change in in p21 protein expression, with combination treatment inducing only a 1.8-fold change in p21 gene expression (Figure 12A, B). Etoposide treatment caused a less than 5-fold change in p21 expression, illustrating the diminished capacity of MDA MB 157 cells to induce p53 pathway activation upon DNA damage compared to HMEC cells (Figure 12B). Western blot data suggests that increased expression and activation of G1 checkpoint proteins upon single and combination drug treatment leads to the G1 accumulation measured in HMEC cells.

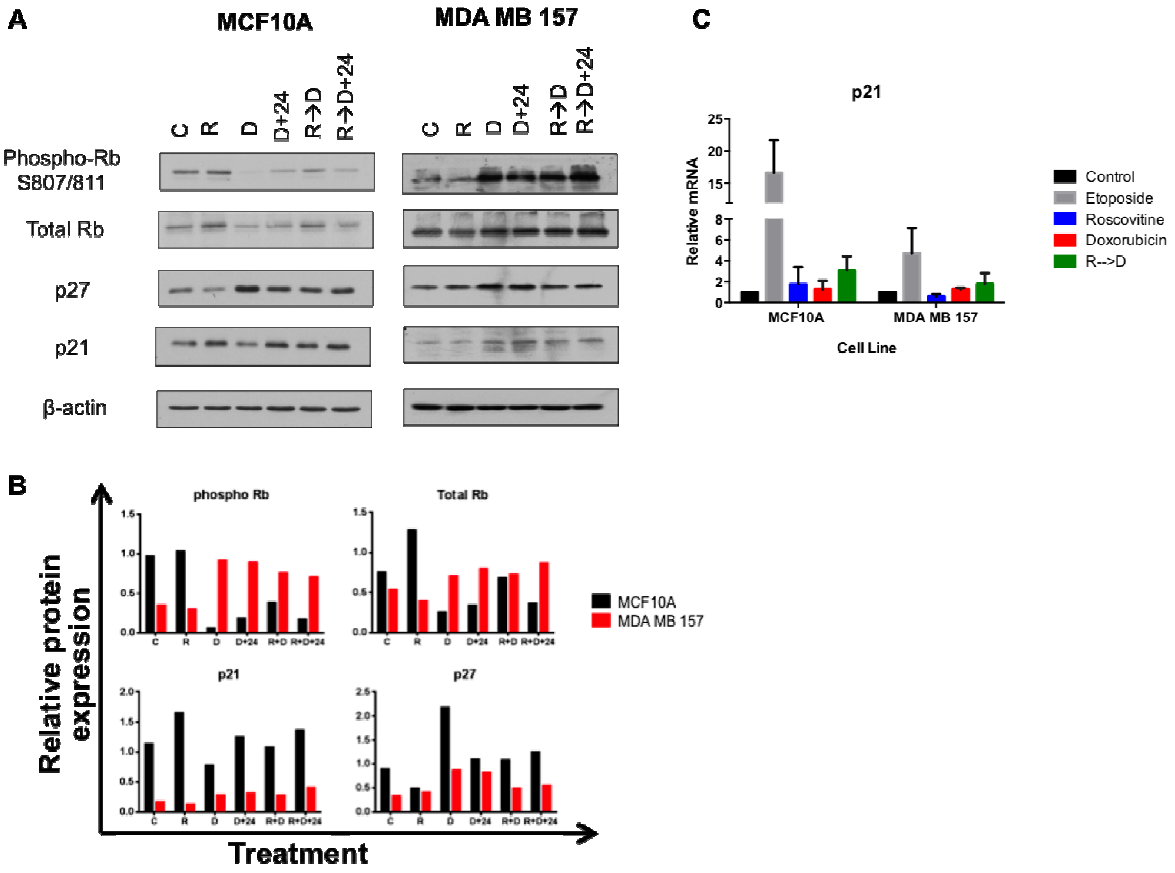


Figure 12. Single and combination treatment activates the G1 checkpoint only in HMEC cells. A, MCF10A and MDA MB 157 cells were subjected to roscovitine for 24 hours (R), doxorubicin for 48 hours (D) and sequential combination treatment (R→D) and harvested as indicated followed by Western blot analysis to detect expression of G1 checkpoint proteins. All drugs were administered at IC_{50} concentrations. B, Densitometry analysis of phospho-Rb, total Rb, p21 and p27 using Image J per cell line. All proteins were normalized to β-actin. C, Cells were subjected to single and combination treatment followed by RNA extraction and qRT-PCR analysis to measure p21 transcription. Etoposide treatment was used as a positive control for p53 activation.

3.3D G1 CHECKPOINT ABLATION CORRELATES TO ROSCOVITINE-DOXOURBICIN SYNERGISTIC RESPONSE IN HMEC CELLS

To examine the effect of G1 checkpoint deregulation in HMEC cells, the immortalized isogenic cell lines 76NF2V and 76NE6 cells were subjected to synergistic combination drug treatment. The viral oncoprotein HPV16-E6 binds and degrades p53 in 76NE6 cells (Table 12) [390]. 76NF2V cells were immortalized with a mutant HPV-16E6 gene (F2V), that is unable to degrade p53, but still immortalizes cells [391]. Sequential administration of roscovitine and doxorubicin caused an additive response in p53 wildtype 76NF2V cells (Figure 13A). However, combination treatment induced synergism in the p53 inactive 76NE6 cells (Figure 13A). These findings suggest that G1 checkpoint deregulation, via p53 degradation or other mechanisms, correlates to response to roscovitine-doxorubicin combination treatment.

To determine how G1 checkpoint regulation affects cell cycle response of HMEC cells to single and combination treatment, 76NF2V and 76NE6 cells were subjected to flow cytometry. Twice as many untreated control 76NE6 cells are in the G2/M phase compared to untreated 76NF2V cells, with 76NE6 cells demonstrating polyploid nuclei. Although doxorubicin induced a G1 accumulation in both cell lines, the percent of 76NE6 cells in G1 decreased by 10% 24-hours post doxorubicin treatment. However, 76NF2V cells continued to have over 75% of cells accumulate G1 24-hours post doxorubicin treatment (Figure 13B). The faster recovery of 76NE6 cells from G1 phase arrest compared to 76NF2V cells suggests that 76NE6 cells are more prone to re-enter the cell cycle following DNA damage. Notably, both cells lines accumulated in almost equal amounts in the G2/M phase due to combination treatment. However, 76NE6 cells had twice as many cells with polyploid nuclei both during and post release from combination treatment compared to 76NF2V cells (Figure 13B). Similar to TNBC cells, the detection of polyploid nuclei in untreated and combination

treated 76NE6 cells suggests that G1 checkpoint deregulation can lead to abnormal DNA content.

To measure expression of G1 checkpoint proteins, 76NF2V and 76NE6 cells were subjected to single and combination treatment followed by Western blot analysis. Roscovitine and combination treatment reduced phospho-Rb expression in 76NF2V cells, with expression returning 24 hours post combination treatment (Figure 13C). The decrease in phospho-Rb caused by single and combination treatment suggests that Rb is inhibiting E2F transcription factors and promoting G1 checkpoint activation. While doxorubicin treatment increased p27 expression, both roscovitine and combination treatment increased p21 expression in 76NF2V cells (Figure 13C). In contrast, roscovitine and combination treatment did not reduce phospho-Rb in 76NE6 cells; however, doxorubicin and 24-hours post release of combination treatment did cause a modest decrease in phospho-Rb expression. Neither single nor combination drug treatment induced p21 expression in 76NE6 cells (Figure 13C). It is expected that 76NE6 cells will be unable to induce p21 expression since p53, the transcription factor for p21, is degraded in these cells. Therefore, Western blot and cell cycle analysis revealed that 76NE6 cells have a reduced capacity to activate their G1-checkpoint.

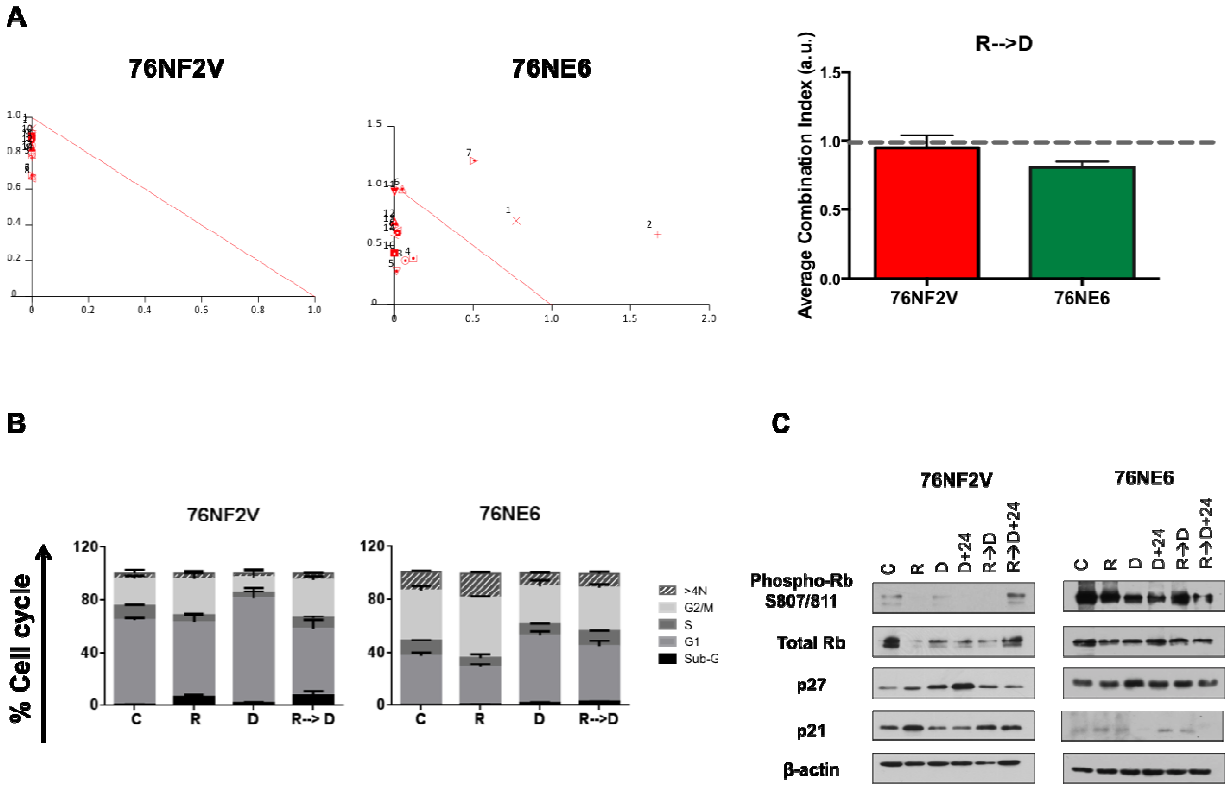


Figure 13. Roscovitine-doxorubicin induced synergism correlates to reduced G1-checkpoint activation. A, 76NF2V and 76NE6 cells were subjected to HTSA where roscovitine and doxorubicin were administered sequentially. Isobolograms and CI values generated with CalcuSyn. B, HMEC cells were treated with roscovitine (R) for 24 hours, doxorubicin (D) for 48 hours and combination treatment (R→D) followed by flow cytometry. Cells were also harvested 24 hours post release from treatment. C, Following single and combination drug treatment, Western blot analysis was used to detect G1 checkpoint proteins in HMEC cells.

3.3E KNOCKDOWN OF RB IS INSUFFICIENT TO INDUCE SYNERGISM IN HMEC CELLS.

Since doxorubicin and combination drug treatment reduced phospho-Rb expression in both MCF10A and 76NF2V cells, but remained elevated in MDA MB 157 cells, Rb was stably knocked down using lentiviral infection in 76NF2V cells to examine the effect of Rb inactivation on combination-induced synergy (Figure 12A and 13C). Western blot analysis was used to confirm knockdown (Figure 14A). When subjected to HTSA followed by CalcuSyn analysis, roscovitine-doxorubicin combination treatment induced additivity or antagonism in non-targeted shScramble or shRb cells, respectively (Figure 14C). Moreover, cell cycle analysis of knockdown cells revealed that ablation of the Rb pathway did not cause 76NF2V cells to accumulate more in the G2/M phase compared to shScramble cells (Figure 14B). Therefore, Rb inactivation is not sufficient to cause roscovitine-doxorubicin-induced synergism in HMEC cells.

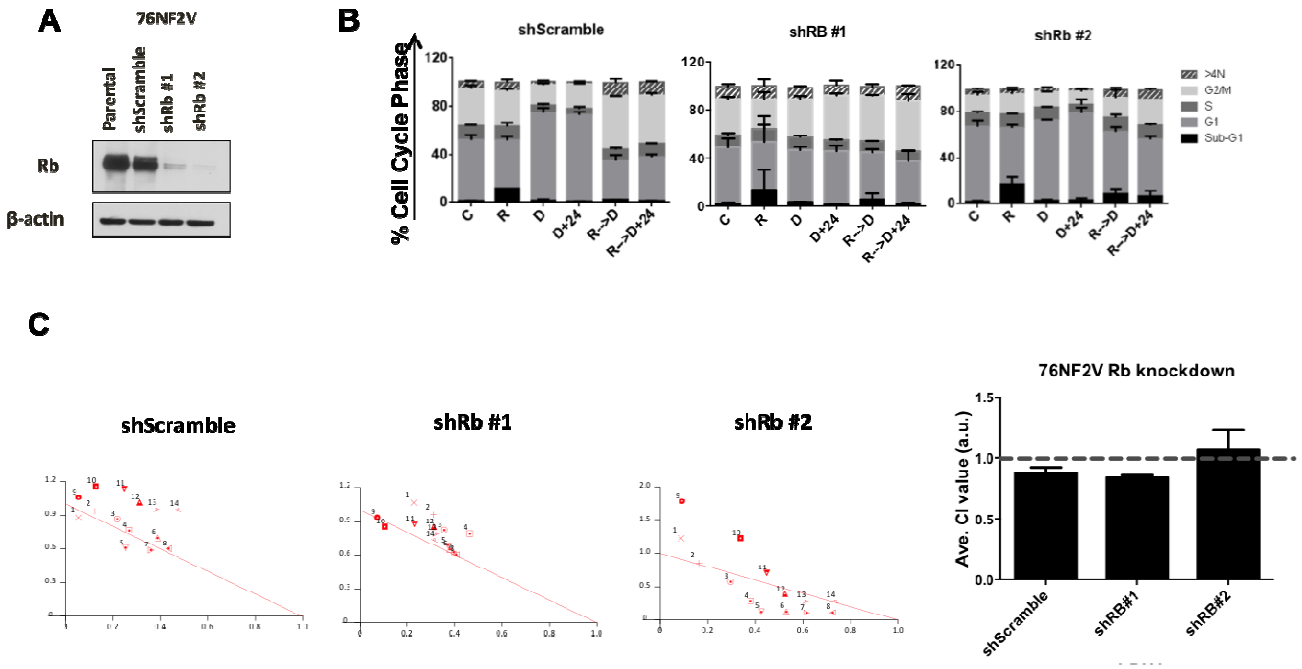


Figure 14. Knockdown of Rb is insufficient to cause HMEC cells to respond synergistically to combination treatment. A, Lentiviral infection was used to generate 76NF2V cells stably expressing non-targeting shRNA (shScramble) or shRNA against Rb (shRB). Western blot analysis was used to confirm knockdown. B, shScrabmble and shRB cells were treated with single (R or D) and combination treatment (R→D) followed by cell cycle analysis. C, 76NF2V shScramble and shRb cells were subjected to sequential roscovitine-doxorubicin combination treatment. Isobolograms and CI values were generated using CalcuSyn.

3.3F KNOCKOUT OF THE P53 PATHWAY CAUSES SENSITIVITY TO ROSCOVITINE-DOXORUBICIN-INDUCED SYNERGISM IN TUMOR CELLS

Thus far, only TNBC and HMEC cells with G1 checkpoint deregulation responded synergistically to roscovitine-doxorubicin treatment (Figure 5A, 13A). However, knockdown of Rb was insufficient to induce synergism in HMEC cells (Figure 14C). Therefore, the direct effect of p53 pathway abolishment in tumors cells was examined in the isogenic colon cancer cells HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$. Western blot analysis confirmed the presence or absence of p53 expression in p53 wildtype and knockout cells, respectively (Figure 15A). When subjected to HTSA and CalcuSyn analysis, p53 wildtype cells responded antagonistically with a CI value of 1.23 to combination treatment. However, knockout of p53 induced synergism in HCT116 cells (Figure 15B). Consequently, abolishment of the p53 pathway is sufficient to sensitize tumor cells to the synthetic lethal roscovitine-doxorubicin combination.

Western blot analysis was employed to examine expression of G1 checkpoint proteins. Sequential roscovitine-doxorubicin treatment reduced phospho-Rb expression in p53 wildtype cells. Moreover, both single and combination drug treatment caused increased expression of p27 and p21 in p53 wildtype cells (Figure 15C). Although both roscovitine and combination treatment caused a reduction in phospho-Rb expression in p53 knockout cells, drug treatment did not increase p27 or p21 expression in these cells (Figure 15C). The inability of HCT116 $p53^{-/-}$ cells to induce p21 expression indicates a compromised G1 checkpoint in these knockout cells.

Cell cycle analysis was performed to examine the effect of combination treatment on p53 wildtype and knockout cells. Although polyploidy was detected in both p53 wildtype and knockout untreated cells, p53 knockout untreated cells had twice as many polyploid nuclei, indicating that knockout of the p53 gene can lead to a propensity for irregular DNA content (Figure 15D). Combination treatment and 24-hours post treatment increased G2/M

accumulation and polyploidy in both p53 wildtype and knockout cells. However, combination-treated p53 knockout cells had twice the amount of Sub-G1 cells (20%) compared to p53 wildtype cells both during and following treatment (Figure 15D). Sub-G1 is indicative of cell death, suggesting that lack of p53 expression correlates to increased cell death in response to roscovitine-doxorubicin treatment in tumor cells. Detecting increased Sub-G1 in HCT116 $p53^{-/-}$ cells due to combination treatment is in accordance with the synergism that was measured using HTSA and CalcuSyn (Figure 15A, D).

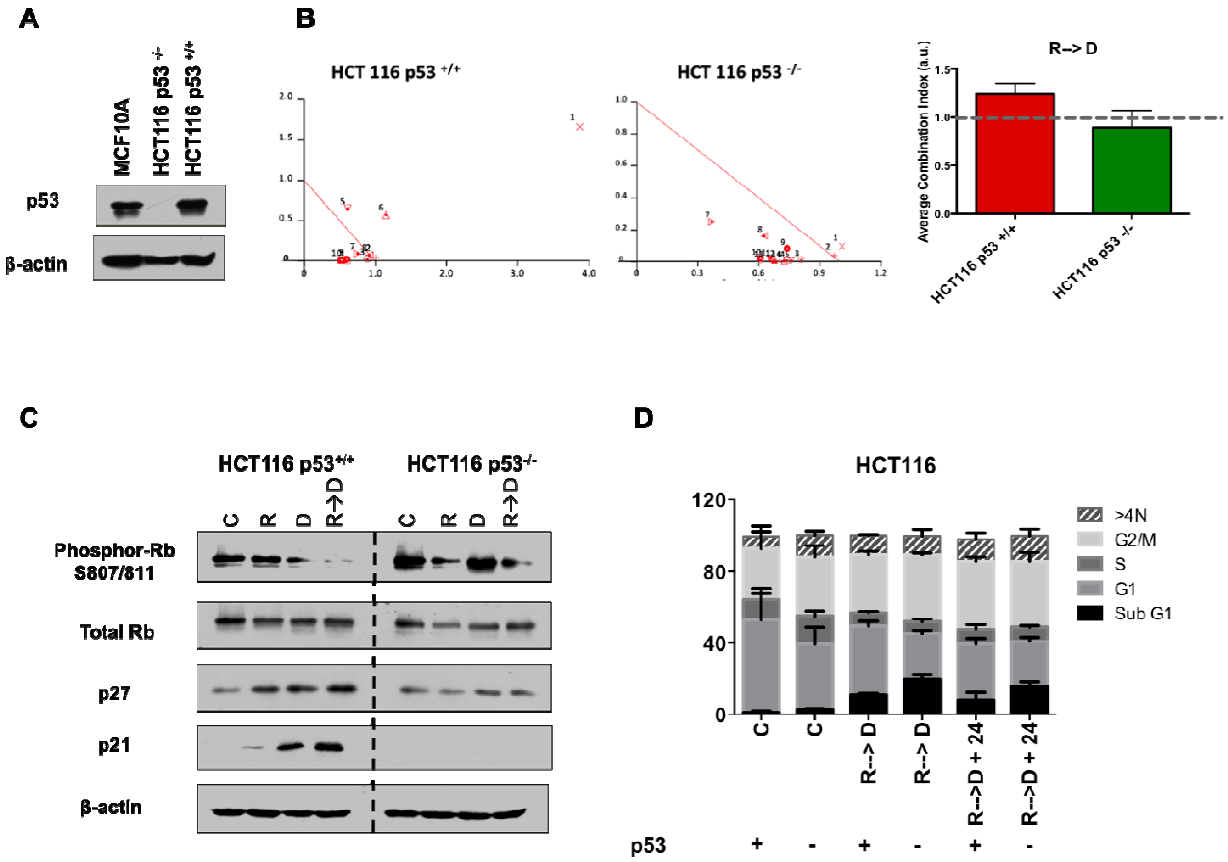


Figure 15. Abolishment of p53 pathway in tumor cells causes synergistic response to roscovitine-doxorubicin combination treatment. A, Western blot analysis confirmed expression and knockout of p53 in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, respectively. MCF10A cells served as a positive control for p53 expression. B, p53 wildtype and knockout cells were subjected to HTSA with sequential roscovitine-doxorubicin treatment followed by CalcuSyn analysis. C, Western blot analysis was used to detect expression of G1 checkpoint proteins. D, p53 wildtype and knockout cells were subjected to flow cytometry following combination treatment and 24-hours post release.

3.3G ROSCOVITINE-DOXORUBICIN TREATMENT INCREASED DNA DSBs SPECIFICALLY IN TNBC CELLS

CDK1 activity is required for recruitment of HR proteins, which repairs DNA DSBs during late S, G2 and M cell cycle phases (Table 16) [112]. Also, pretreatment with roscovitine inhibits recruitment of HR protein RPA34 to damage sites [362]. Thus, we performed a neutral comet assay to quantify DNA DSBs in response to single and combination drug treatment. The tail moment, or the amount of DNA in the distance traveled, measures the extent of DNA DSBs. As expected, treatment with doxorubicin induced DNA DSBs, indicated by an increased tail moment, in both HMEC and TNBC cell lines (Figure 16). However, administering roscovitine prior to doxorubicin caused a significant (p value < 0.05) increase in the amount of DNA DSBs only in TNBC cells (Figure 16). Combination treatment did not cause an increase in DNA DSBs in MCF10A cells (Figure 16). This indicates that treating TNBC cells with roscovitine prior to doxorubicin can enhance the DNA damage inflicted by doxorubicin.

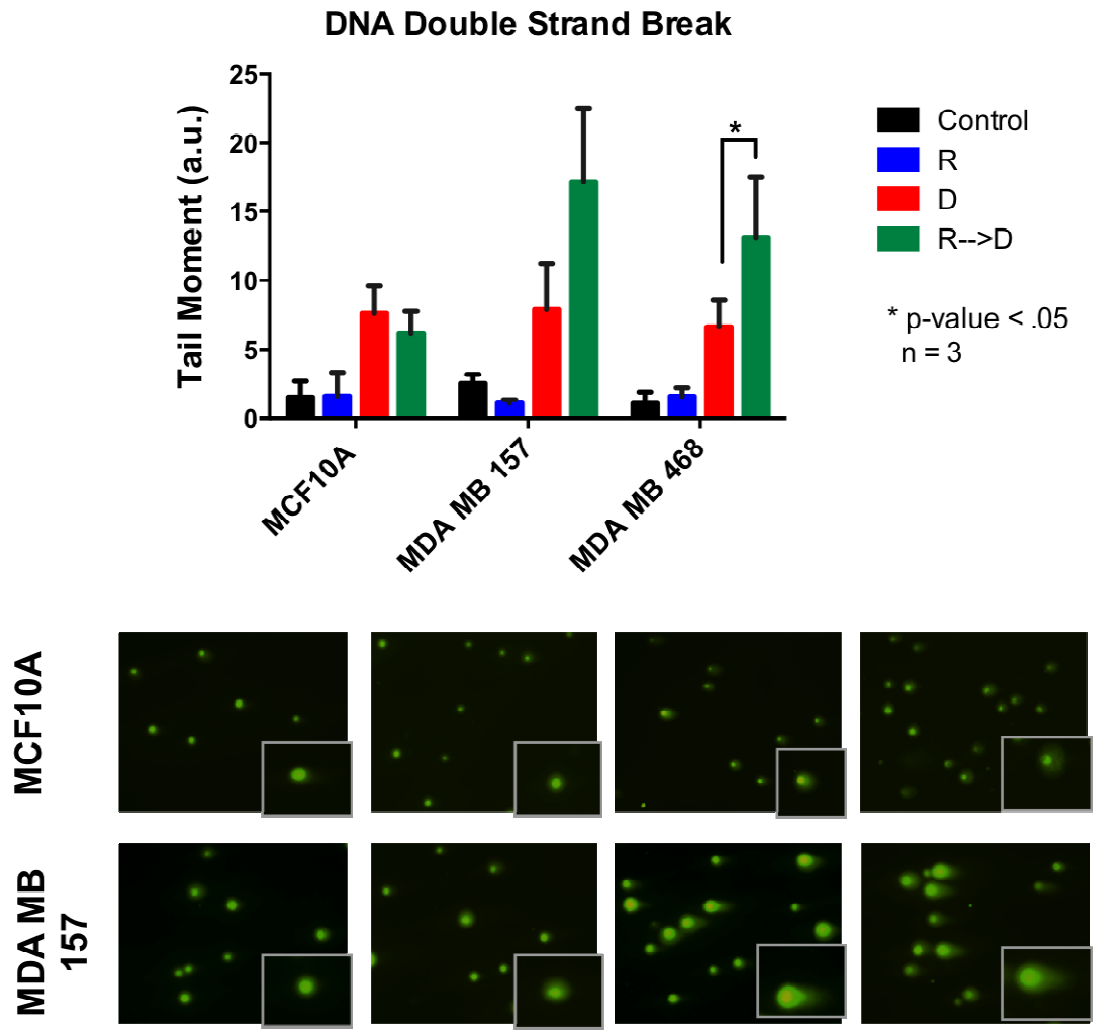


Figure 16. Administering roscovitine prior to doxorubicin increases DNA DSBs explicitly in TNBC cells. HMEC and TNBC cells were treated with roscovitine (R), doxorubicin (D) or combination drug treatment (R→D) and subjected to the neutral comet assay. Images were captured at 10x. Tail moment was measured using Comet Score.

3.3H COMBINATION TREATMENT INCREASES DNA DSBs WHILE REDUCING RECRUITMENT OF DOWNSTREAM HR PROTEINS

Due to the increase in DNA DSBs we measured in TNBC cells, γ-H2AX foci, a marker of DNA DSBs, was examined in response to single and combination treatment. Doxorubicin treatment induced γ-H2AX foci in about 60% of MCF10A cells and both TNBC cell lines (Figure 17A, B, C). However, treatment with roscovitine prior to doxorubicin significantly increased (p-value < 0.05) the percentage of γ-H2AX positive cells by 20% explicitly in TNBC cells (Figure 17B,C). Combination treatment did not cause an increase in DNA damage in MCF10A cells (Figure 17A, C). Indeed, combination treated MDA MB 157 cells had significantly 20% more (p-value < 0.05) γ-H2AX positive cells than combination treated MCF10A cells (Figure 17C). The increase in γ-H2AX foci only in TNBC cells confirms that combination treatment causes more DNA DSBs than doxorubicin treatment alone.

As previously shown, combination treatment causes accumulation of TNBC cells in the G2/M cell cycle phase, where cells rely on HR for DSB repair (Figure 10) [105]. The HR protein Rad51 binds to the excised single strands to facilitate strand invasion (Figure 2). Quantification of γ-H2AX positive cells with Rad51 foci was used to examine the recruitment of downstream HR proteins. Untreated control HMEC and TNBC cells had limited DNA damage, but had over 60% or over 80% cells with Rad51 positivity when γ-H2AX was present, respectively (Figure 17A, B, D). Roscovitine treatment reduced the recruitment of Rad51 in both MCF10A and MDA MB 157 cells. Moreover, combination treatment significantly (p-value < 0.05) reduced the formation of Rad51 foci by 20% compared to doxorubicin treatment in TNBC cells, even though over 80% of these cells had γ-H2AX positivity (Figure 17B, D). Combination and doxorubicin only treated MCF10A cells were able to recruit Rad51 foci at a similar percentage of 35% (Figure 17A, D). However, over

70% of these cells are in G1, and thus do not rely on HR for DSB repair (Figure 10A, B). These findings are consistent with previous studies demonstrating that roscovitine decreased RPA34 foci in response to irradiation despite formation of γ-H2AX foci [362]. Therefore, treatment with roscovitine prior to doxorubicin impaired TNBC cell DNA damage response to DNA DSBs, regardless of increased DNA damage and cell cycle phase.

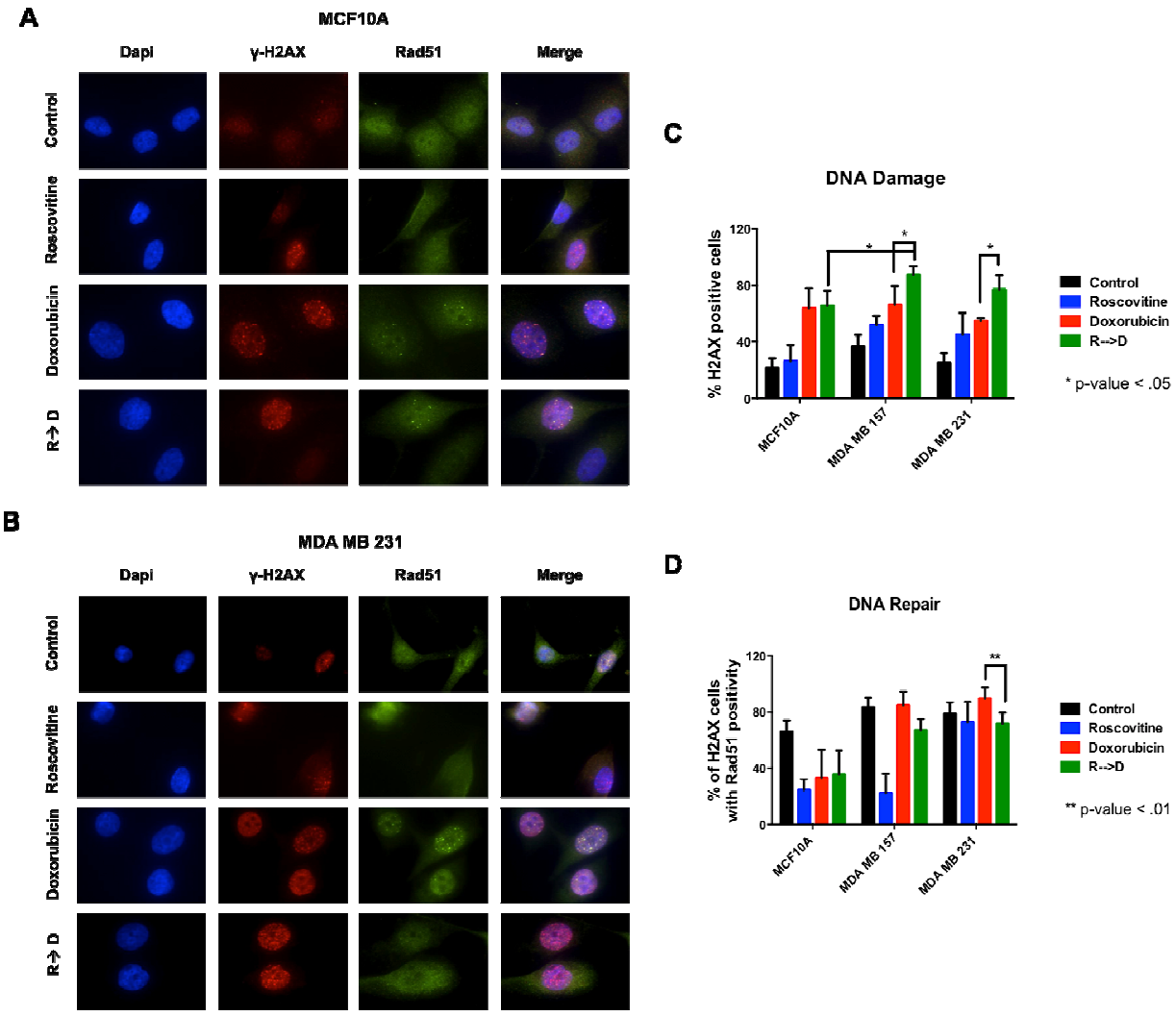


Figure 17. Roscovitine-doxorubicin treatment increases DNA DSBs while reducing recruitment of downstream HR proteins in TNBC cells. A and B, MCF10A and MDA MB 231 cells were treated with single and combination drug treatment followed by immunofluorescence to detect γ-H2AX and Rad51 foci. C, Percent of cells with γ-H2AX were quantified in HMEC and TNBC cells. Cells with ≥5 foci were considered positive. D, Percent of γ-H2AX-positive cells with Rad51 positivity. Cells with ≥1 foci were considered positive. One hundred cells per sample were counted per trial (n ≥ 3).

3.3I ABSENCE OF P53 ALLOWS COMBINATION TREAMENT TO INCREASE DNA DSBs

HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ were used to determine the effect of p53 pathway abolishment on DNA damage and repair. Presence or absence of p53 had little effect on doxorubicin-induced DNA damage, with 41% and 46% of p53 wildtype and p53 knockout cells having γ-H2AX positivity, respectively (Figure 18A, B, C). However, combination treatment significantly increased (p-value < 0.01) γ-H2AX positive cells to 60% only in p53 knockout cells compared to doxorubicin treated cells. Combination treatment did not augment γ-H2AX positivity in p53 wildtype cells. Moreover, combination treated p53 knockout cells had significantly 20% more (p-value < 0.01) γ-H2AX positive cells compared to combination treated p53 wildtype cells (Figure 18C). As such, absence of p53 causes tumor cells to have increased DNA damage in response to combination treatment.

The effect of deletion of p53 on DNA DSB repair through the HR pathway was measured through the recruitment of Rad51 foci in γ-H2AX positive cells. Upon doxorubicin treatment, p53 wildtype and p53 knockout cells recruited Rad51 to γ-H2AX sites in 63% and 59% of cells, respectively (Figure 18A, B, D). Combination treatment had no effect on Rad51 recruitment in p53 wildtype cells. However, p53 knockout cells had a significant decrease (p-value < 0.05) in the percent of γ-H2AX positive cells with Rad51 foci compared to doxorubicin treated cells (Figure 18A, B, D). Despite having more G2/M cells, p53 knockout cells had almost 20% less recruitment of Rad51 foci than p53 wildtype cells during combination treatment (Figure 15D, Figure 18A, B, D). Therefore, deletion of p53 reduces the ability of tumor cells to recruit downstream HR proteins during roscovitine-doxorubicin combination treatment.

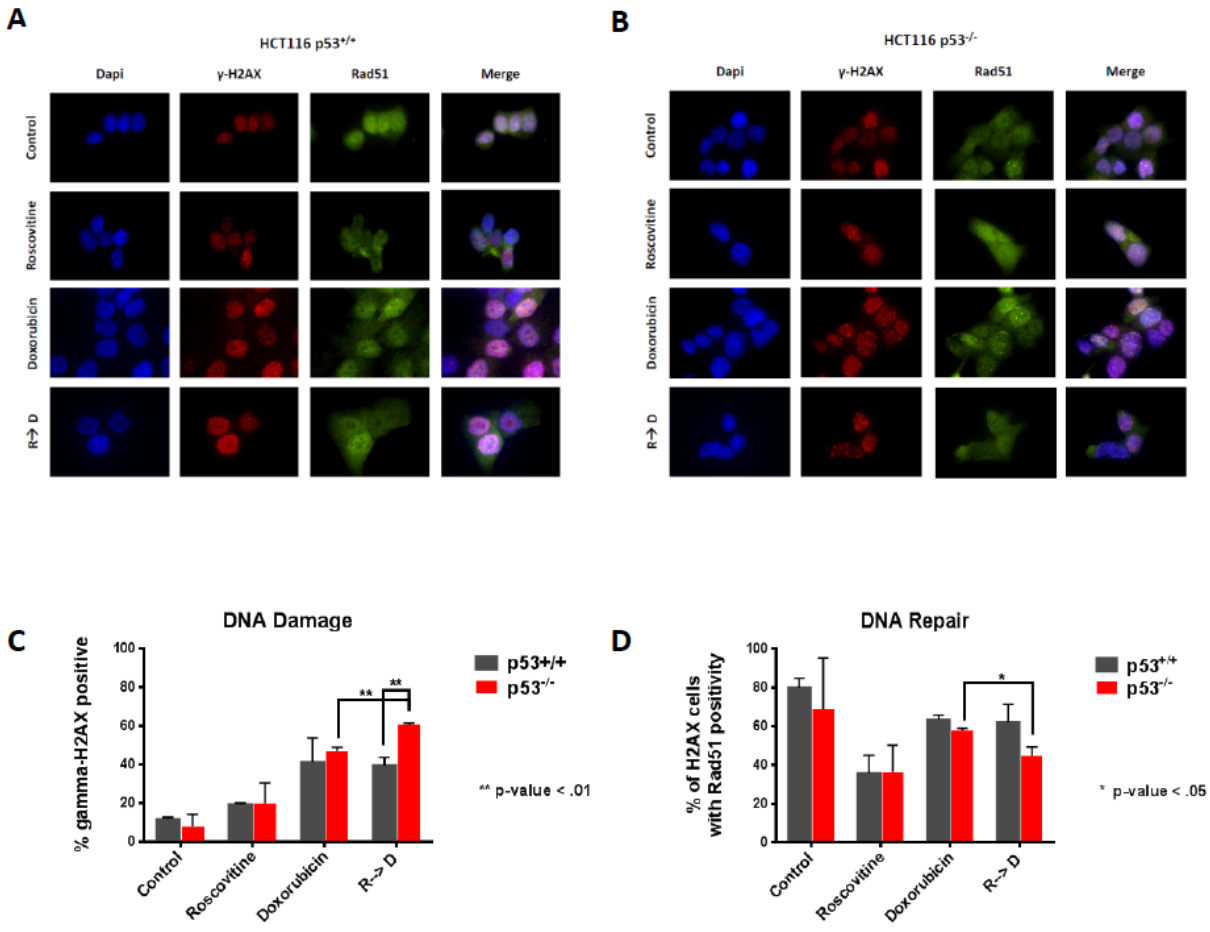


Figure 18. Abolishment of p53 pathway causes combination treatment to increase DNA DSBs. A, B HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were subjected to single and combination drug treatment followed by immunofluorescence to detect γ-H2AX and Rad51 foci. C, Percent of γ-H2AX positive cells were quantified. Cells with ≥5 foci were considered positive. D, Percent of γ-H2AX positive cells with Rad51 foci quantified. Cells with ≥1 foci were considered positive. At least one hundred cells per sample were counted per trial

3.4 CONCLUSIONS

Roscovitine-doxorubicin combination treatment is synthetic lethal explicitly in tumor and HMEC cells with an abrogated p53 pathway. The role of p53 inactivity in sensitizing cells to combination treatment was addressed using isogenic HMEC and tumor cells with and without p53 activity. Here, loss of p53 at the protein or genomic level, and concordant lack of p21 expression, lead combination treatment to be synthetically lethal. Cells with p53 activity were consistently less sensitive to combination treatment. Moreover, loss of Rb did not sensitize HMEC cells to combination treatment. Therefore, G1 checkpoint deregulation via p53 inactivity is required for roscovitine-doxorubicin-induced synergism in TNBC cells.

Loss or mutated p53 is associated with increased accumulation in the G2/M cell cycle phase and polyploid nuclei in untreated, single and combination treated cells. HMEC and tumors cells with an intact p53 pathway are more prone to arrest or accumulate in the G1 cell cycle phase in response to treatment. Whereas knockdown of CDK1 is sufficient to cause accumulation of cells in G2/M arrest in the presence of doxorubicin in TNBC cells, p53 wildtype HMEC cells continued to accumulate in the G1 phase during these conditions. Moreover, ablation of the Rb pathway did not increase G2/M phase accumulation in HMEC cells. These findings demonstrate that p53 pathway abrogation allows TNBC cells to bypass the G1 checkpoint, causing increased G2/M accumulation and potentially irregular DNA content in response to combination therapy.

The role of p53 is not limited to the G1 checkpoint, as p21 also inhibits CDK activity during G2 and M phases (Figure 1). DNA damage can cause p53 to arrest cells in G1 or G2 [84]. Irradiation of HCT116 $p53^{-/-}$ cells demonstrated that p53 is required for G2 arrest maintenance, with knockout cells entering mitosis and failing cytokinesis [392]. Therefore, the observation of polyploid nuclei in untreated and treated p53 mutant TNBC cells may also be due to a compromised G2 checkpoint that allows cells to progress through mitosis regardless of damaged DNA.

Combination treatment caused increased DNA DSBs in TNBC cells while reducing the recruitment of downstream HR proteins. Arresting TNBC cells in the G2/M cell cycle phase via CDK inhibition primes them for DNA damage assault. Cells rely on HR to repair DNA DSBs during late S, G2 and M phases of the cell cycle. Inhibition of CDK1 impairs DNA end resection and recruitment of HR proteins, potentially forcing cells to rely on more error-prone methods (i.e. MMEJ) for repair or leaving breaks unrepaired. Thus, by inducing a G2/M accumulation via CDK inhibition followed by doxorubicin treatment, TNBC cells are being subjected to DNA damage while having a compromised HR pathway. Moreover, p53 pathway abolishment enabled combination treatment to increase DNA DSBs while inhibiting recruitment of HR proteins in tumor cells. The conclusion that TNBC and other tumor cells that are accumulated in the G2/M phase have increased sensitivity to doxorubicin treatment is consistent with previous studies that found doxorubicin induced more cytotoxicity in mouse-derived leukemia p53-mutant cells when they were synchronized to S and G2/M [393]. In the present study, non-transformed cells with an intact p53 pathway are protected from the most damaging effects of doxorubicin because they arrest in G1 phase. p53 wildtype tumor cells were also protected from additional DNA damage. Therefore, upon combination treatment, TNBC cells undergo increased DNA damage with compromised DNA DSB repair pathways; HMEC cells remain relatively unharmed.

The concept of chemoprotecting normal tissues from cytotoxic drugs by inducing a reversible cell cycle arrest is called cyclotherapy [394]. This concept is especially promising for targeting p53 mutant tumors. Current studies have utilized p53 activators antinomycin D and nutlin-3, a MDM2 inhibitor, to activate p53 and arrest normal cells, shielding them from S and M phase toxins [395]. For example, treatment with nutlin-3 protected p53 competent tumor cells U2OS and HCT116 $p53^{+/+}$ and non-transformed human keratinocytes from gemcitabine treatment. However, pre-treatment with nutlin-3 did not arrest HCT116 $p53^{-/-}$, allowing gemcitabine to remain cytotoxic in these cells [396].

Both single and combination treatment induced G1 arrest that correlated to p53 activity, as measured by p21 expression, in p53-competent HMEC and tumor cells. Activation of p53 protected these cells from doxorubicin-associated cyto-toxicities. The shielding of non-transformed cells via p53 activation supports the finding that doxorubicin treatment was more toxic *in vivo* than combination treatment (Figure 9C,D and Table 15). Also, administering roscovitine first is also advantageous because it further sensitized TNBC cells to doxorubicin treatment. The requirement of abrogated p53 activity to cause combination-induced synergism enables specific targeting of p53-mutant TNBC cells while simultaneously limiting the damage inflicted on non-transformed cells.

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

4.1 MAJOR FINDINGS

TNBC is an aggressive disease associated with decreased overall survival in which tumors are molecularly and clinically distinct from non-TNBC tumors. Since patients with TNBC tumors cannot benefit from clinically available targeted therapies, there is an urgent need to develop novel treatment strategies that target the specific biology of TNBC tumors. Due to a majority of TNBC tumors carrying mutations in the cell cycle machinery, including p53, Rb and cyclin E, it was hypothesized that **TNBC cells are sensitive to cell cycle targeted combination therapy, leaving non-transformed cells unharmed.** The data presented in this dissertation addressed the questions posed in the first chapter.

1. Would the deregulation of the cell cycle sensitize TNBC cells to cell cycle inhibitors; would non-TNBC and non-transformed cells react differently?

Treatment with roscovitine induced a significant G2/M arrest in TNBC cells (all p53-mutant) and in the HMEC cell line 76NE6 cells, which does not express p53. Roscovitine treatment did not induce a major G2/M arrest in HMEC and ER-positive cells with intact (or heterozygous) p53 pathways. Therefore, sensitivity to cell cycle inhibitors is sub-type and p53-mutant specific.

2. Can a synthetic lethal combination of cell-cycle inhibition and chemotherapeutics specifically target TNBC cells, without inflicting harm to non-transformed cells?

Sequential administration of roscovitine and doxorubicin treatment was synthetic lethal specifically in TNBC cells, but was antagonistic in HMEC cells. Notably, simultaneous drug administration or treating with doxorubicin prior to roscovitine was antagonistic in TNBC cells. Combination treatment prolonged and increased apoptosis only in TNBC cells compared to single drug treatments.

3. Will combination cell cycle targeted therapy be an effective method in a pre-clinical model?

Combination treatment was both efficacious and well tolerated in mice with human TNBC xenografts. Since combination treatment inhibited tumor growth without increasing toxicity, no combination treated mice were euthanized, resulting in a significant increase in overall survival. Despite being well tolerated, roscovitine had very limited efficacy, causing all mice to be euthanized due to excessive tumor burden. Mice treated with doxorubicin suffered from excessive tumor burden and deteriorated health, illustrating its inadequacy as a single agent.

4. What affect would combination therapy have on the cell cycle of TNBC cells compared to non-transformed cells?

Both single and combination treatment maintained a G2/M arrest and/or induced polyploid nuclei specifically in TNBC cells. In contrast, HMEC cells accumulated in the G1 phase in response to treatment. Moreover, knockdown of CDK1 was sufficient to augment the percent of G2/M accumulation in the presence of doxorubicin only in TNBC cells.

5. Is there a molecular target/pathway that can be used as a marker to predict combination treatment response?

TNBC cells with p53 mutations had a diminished capacity to induce p21 transcription compared to p53 wildtype HMEC cells. Knockdown of Rb was insufficient to cause a synergistic response in HMEC cells. However, combination treatment was synthetic lethal in p53-compromised HEMC and tumors cells, whereas the paired isogenic p53 wildtype cells were additive or antagonistic to treatment. Moreover, the cell cycle profile of HMEC and tumor cells lacking p53 activity closely mimicked the cell cycle profile of TNBC cells; the isogenic p53 wildtype cells had less G2/M accumulation. Thus, p53 inactivation serves as a putative predictor of synergistic response to roscovitine-doxorubicin combination treatment.

6. Can combination treatment augment the DNA damage inflicted by chemotherapeutics explicitly in TNBC cells?

Administration of roscovitine prior to doxorubicin caused increased DNA DSBs while reducing the recruitment of the downstream HR protein Rad 51 in TNBC cells. Combination treatment did not subject HMEC cells to increased DNA damage. Moreover, combination treatment increased DNA damage and reduced recruitment of HR proteins in p53-null tumor cells compared to isogenic p53-wildtype cells. Therefore, ablation of the p53 pathway in TNBC cells can further sensitize cells to combination treatment-associated DNA damage.

4.2 LIMITATIONS

The present study focused on increasing the innate sensitivity of TNBC cells to anthracycline-based therapy. However, TNBC tumors are also sensitive to platinum-based chemotherapies, such as cisplatin. Since platinum-based therapies also induce DNA damage by binding to DNA purine bases, it is reasonable to hypothesize that roscovitine treatment could synergize with cisplatin. Additionally, because anthracyclines are associated with cardiotoxicity, it would be beneficial to determine how well tolerated roscovitine and platinum-based chemotherapy combination treatment is.

4.3 FUTURE DIRECTIONS

The roscovitine-doxorubicin combination is a novel treatment strategy that can specifically target p53-mutated TNBC tumors. However, TNBC is a heterogeneous disease and identifying the population of patients that will benefit the most from combination treatment, while preventing unnecessary treatment of non-responder patients, is necessary for effectively implementing this therapy. The TNBC cell lines used in this study, including MDA MB 157, MDA MB 231 and MDA MB 468 have a range of p53 mutations. MDA MB 157 cells have a 26 base pair deletion in codon 261, MDA MB 231 cells have a missense mutation in codon 280 and MDA MB 468 cells have a missense mutation in 273, all of which occur in the DNA binding domain of p53 [348]. However, p53 is deregulated through multiple mechanisms, including missense mutations, deletions, GOF mutations, MDM2 overexpression and deletions in p14 (a negative regulator of MDM2). The effect of p53

status on treatment response has been inconsistent. As previously described, p53 mutations in the zinc binding domain conferred resistance to doxorubicin treatment [373]. Moreover, Bourdon et al. found that the p53 gene has an alternative internal promoter that can transcribe 9 splice variants with differing functions. For example, variant p53β can heighten p53 target gene expression, whereas variant Δ133p53 acts as a dominant-negative and inhibits p53 full-length activity. These isoforms were differentially expressed between normal versus tumor tissue [397]. These splice variants potentially contribute to the discrepancies between p53 status and treatment response [348, 397]. The effects of these splice variants and other p53 mutations have not been directly investigated in the present study. Since HCT116 $p53^{-/-}$ cells were synergistic to combination treatment, it can be hypothesized that MDM2 signaling for p53 degradation would induce synergism in tumor cells. However, it is undetermined whether MDM2 overexpression or p14 gene deletions are sufficient to sensitize tumor cells to combination treatment. Therefore, further investigation is required to examine the effect of differing p53 mutations and pathway deregulations on combination treatment response. As a result, it is likely that patient tumors will have to be sequenced to distinguish responders from non-responders.

Roscovitine-doxorubicin treatment was well tolerated in an *in vivo* model system. However, a phase I study examining the MTD and efficacy of dinaciclib in combination with the anthracycline epirubicin found that this treatment was very toxic, ending the trial before efficacy could be determined. Dose-limiting toxicities included neutropenia, syncope and vomiting (NCT01624441). To increase the efficacy of combination treatment while reducing toxicity, the drugs could be paired with a nanocarrier delivery system. Coupling roscovitine or doxorubicin with nanotechnology drug deliverance system will limit dispersal of the drug to only at the site of action, protecting other organs and tissues from cytotoxicity [398]. For example, anti-HER2 immunoliposomes containing doxorubicin were targeted to HER2 overexpressing tumors, increasing the therapeutic benefit of doxorubicin while reducing

toxicity in a xenograft mouse model [399]. Roscovitine could be directly delivered to the tumor site if it was bound to a ligand-mediated active binding nanoparticle. EGFR, which is overexpressed in a majority of TNBC tumors, is a cell surface receptor that provides a putative target to deliver roscovitine to the tumor site. Indeed, EGFR-targeted polymer nanocarriers delivered paclitaxel and ionidamine to multi-drug resistant EGFR-overexpressing tumor cells, increasing drug cytotoxicity [400]. Developing EGFR-targeted nanocarriers to deliver to roscovitine directly to the tumor site could increase the therapeutic benefit of CDK inhibition while reducing toxicities. Moreover, liposomal-doxorubicin, which accumulates at tumor sites due to leaky vasculature, is clinically available to treat breast cancer [401]. Thus, it would be clinically beneficial to consider incorporating roscovitine-doxorubicin combination treatment with nanoparticle delivery system.

Investigating the application of this combination treatment in other types of cancers was beyond the scope of the present study. However, p53 is mutated in over 50% of cancers. Previous studies in our laboratory found that roscovitine-doxorubicin combination treatment induce synergism in sarcoma cells [343]. Additionally, this study found that combination treatment was synergistic in the colorectal cancer cell line HCT116 $p53^{-/-}$. Furthermore, pretreatment with roscovitine could enhance the cytotoxicity in the broad range of malignancies that doxorubicin is currently used to treat, including bladder, head and neck, liver, lung, ovarian cancer and sarcomas [254]. As such, the therapeutic benefit of this novel combination treatment strategy may extend beyond TNBC. Future studies should assess the efficacy of roscovitine-doxorubicin combination treatment in other cancers with p53 pathway deregulation.

4.4 SIGNIFICANCE

TNBC patients have no therapeutic options beyond chemotherapy and surgery. Due to the aggressive nature of TNBC and decreased overall survival of these patients, there is a pressing need to develop innovative treatment strategies that target the molecular and

biological characteristics attributed to TNBC cells. Here, **sequential administration of roscovitine prior to doxorubicin is synthetic lethal explicitly in TNBC cells, with non-transformed cells remaining unharmed**. Demonstrating promise in a pre-clinical *in vivo* model, this combination strategy can greatly impact the treatment and care of TNBC patients. Since the success of this combination treatment requires p53 pathway abrogation, mutated p53 status provides a putative predictor of response. Overall, roscovitine-doxorubicin combination could potentially become a powerful tool for clinicians to treat TNBC.

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